BARD Final Report US-4771-14R

Uptake of wastewater-derived micropollutants by plants irrigated with reclaimed wastewater

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Project award year: 2014
Three year research project

Irrigation with reclaimed wastewater is increasingly practiced in arid and semi-arid areas of the U.S. and in Israel. This practice offers numerous benefits to agriculture, and its importance is expected to increase as the demand for food grows with global population. However, the presence of certain organic pollutants in reclaimed wastewater may pose risks to human health and the environment when used for irrigation. Conventional wastewater treatment processes are only partially effective in removing a variety of chemicals of emerging concern, including pharmaceuticals and personal care product ingredients (PPCPs). These contaminants have been detected in reclaimed wastewater, reclaimed wastewater-irrigated soils, biosolids, biosolids-amended soils, surface water and groundwater. Some PPCPs can be taken up by crop plants, but little is known about the mechanisms of their uptake and translocation to different plant components, the physicochemical properties of contaminants that affect these processes, and the effects of soil properties and management practices on the uptake of these compounds and their accumulation within plant tissues.

We aimed to systematically evaluate the bioaccumulation of PPCPs with contrasting chemical characteristics by crop species as well as by the model plant *Arabidopsis thaliana*. Our objectives were to determine: (1) the influence of chemical properties on uptake and translocation pathways in selected plants; (2) the effects of plant-induced changes to the rhizosphere on PPCP availability and accumulation; (3) *in planta* metabolism of wastewater-derived micropollutants; and (4) the effects of soil properties on chemical availability, uptake, and distribution in selected plants. We have shown that chemical molecular descriptors alone have limited ability to adequately predict uptake/bioaccumulation and predictive efforts using existing data may be hindered by inadequate understanding of metabolism *in planta*, effects of contaminants on plants, and effects of plant-driven environmental changes on phytoavailability. We found that accumulation, mixture effects, and metabolism varied among crop species. Water uptake by the plants correlated with CBZ and lamotrigine (LTG) loss from solution across species, indicating that the loss of the compounds is likely due to transformation *in planta*. Our data demonstrate the importance of considering species differences when investigating plant accumulation and metabolism of pharmaceuticals, and caution that model species may not be representative of important crop species. We examined the effects of plant-driven rhizosphere pH changes on trends in wheat accumulation of both an ionizable and a non-ionizable contaminant to discriminate between effects on accumulation due to changes in rhizosphere pH and those due to other changes to the plants caused by differences in nutrient availability. We found that plant-driven changes in rhizosphere pH alter accumulation of LTG (pK_a of conjugate base = 5.7), but do not affect accumulation of CBZ, a non-ionizable contaminant. The amount of neutral LTG available in pore water correlated strongly with accumulation in roots and above-ground tissues. Future studies on plant uptake of PPCPs should consider rhizosphere pH separately from bulk soil pH and report concentrations of nutrients available to the plants. We found root exudates alter LTG sorption to soils with low, but not high, organic carbon contents. We showed that fluoxetine and amitriptyline decreased the accumulation of the primary and potentially toxic metabolite of carbamazepine, 10,11-epoxycarbamazepine. Transpiration was strongly correlated with the accumulation of the PPCPs studied. Accumulation of CBZ and LTG, but not naproxen and ketoprofen were reduced due to association with dissolved organic matter in irrigation water. Compound hydrophobicity (as expressed by the *n*-octanol-water distribution ratio) and pK_a strongly influenced compound uptake.

Summary Sheet

Publication Summary

PubType	IS only	Joint	US only
Abstract - Presentation	0	0	4
Reviewed	0	0	1
Submitted	0	0	1
Thesis - Ph.D.	1	0	1

Training Summary

Trainee Type	Last Name	First Name	Institution	Country
Ph.D. Student	Nason	Sara	University of Wisconsin - Madison	USA
Ph.D. Student	Miller	Elizabeth	University of Wisconsin - Madison	USA
Ph.D. Student	Malchi	Tomer	HUJI	Israel
Ph.D. Student	Goldstein	Myah	HUJI	Israel
M.Sc. Student	Mordechay	Evyatar	HUJI	Israel

Contribution of the Cooperation

The collaboration between the investigators at Hebrew University of Jerusalem (HUJI) and University of Wisconsin – Madison (UW) allowed both groups to focus on a subset of pharmaceutical compounds to design and conduct complementary experiments at the two institutions and to use similar analytical methods. The HUJI group provided valuable input on the review article published by the UW group,[1] and we are preparing a joint manuscript describing our work on species differences in plant accumulation and metabolism of pharmaceuticals for submission to *Environmental Science and Technology*. The UW graduate students benefitted from visiting HUJI near the beginning of the joint project. This allowed us to better coordinate our experimental and analytical approaches. The graduate students at both institutions gained much from regular interaction over the course of the BARD project. The graduate students from both groups held periodic meetings over Skype and exchanged emails regarding joint projects. Profs. Pedersen, Shenker and Chefetz, as well as the graduate students, met at international conferences to discuss project progress and joint publications. The sharing of ideas between the two groups strengthened the work conducted at both institutions.

RESEARCH ACHIEVEMENTS

Species Differences in Plant Accumulation and Metabolism of Pharmaceuticals

The University of Wisconsin – Madison (UW) and Hebrew University of Jerusalem (HUJI) collaborated closely to examine plant species differences in the accumulation and metabolism of pharmaceuticals. Pharmaceuticals are commonly detected contaminants in treated wastewater, which may be used to irrigate food crops in arid regions.[1] Approaches to predict plant uptake of pharmaceuticals are necessary, because monitoring produce for all potential contaminants is impractical. However, the variation among plant species in the uptake, translocation, and metabolism of pharmaceuticals is not well understood. We grew *Arabidopsis thaliana*, spinach (*Spinacia oleracea*), cucumber (*Cucumis sativus*), and tomato (*Solanum lycopersicum*) plants and exposed them to CBZ and LTG alone and in combination. We found that accumulation, mixture effects, and metabolism varied among species. Cucumber and tomato accumulated higher concentrations of CBZ metabolites (**Fig. 1**) and a significantly lower percentage of the initial compound provided was detected in the nutrient solution and plants at the end of the exposure period than for spinach and *A. thaliana* (**Fig. 2**). Water uptake by the plants correlated with CBZ and LTG loss from solution across species, indicating that the loss of the compounds is likely due to transformation *in planta*. This hypothesis is supported by higher degree of similarity between human drug metabolism enzymes and those in tomato and cucumber than those in spinach and *A. thaliana* (**Table 1**). We further hypothesize that photodegradation of CBZ and LTG in plant leaves occurred to a larger extent in the cucumber and tomato plants. Our data demonstrate the importance of considering species differences when investigating plant accumulation and metabolism of pharmaceuticals, and caution that model species may not be representative of important crop species. A manuscript describing this work is in preparation.

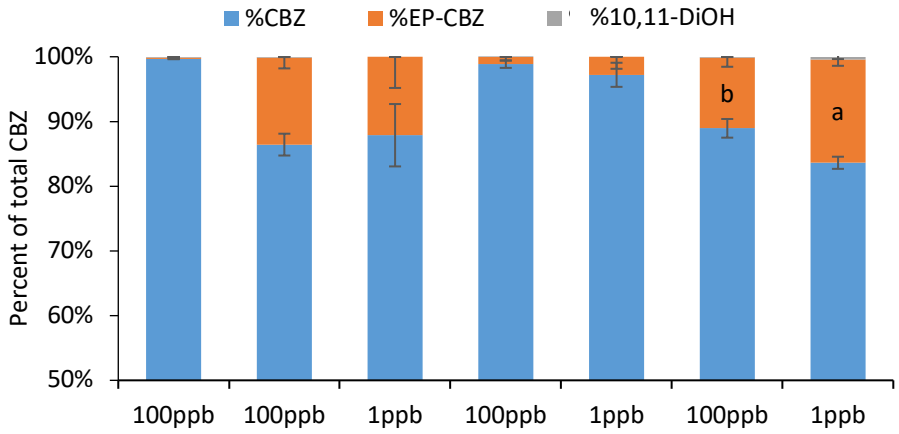

Figure 1. Distribution of carbamazepine and metabolites, 10,11-epoxycarbamazepine (EP-CBZ) and 10,11-dihydroxycarbamazepine (10,11 DiOH), in the leaves of different crops. Metabolite accumulation was very low in *A. thaliana* and spinach. More metabolites accumulated in the 1 $\mu g \cdot L^{-1}$ tomato exposed leaves than the 100 $\mu g \cdot L^{-1}$ exposed leaves. Note that the y-axis begins at 50%. Error bars represent one standard deviation ($n \geq 3$).

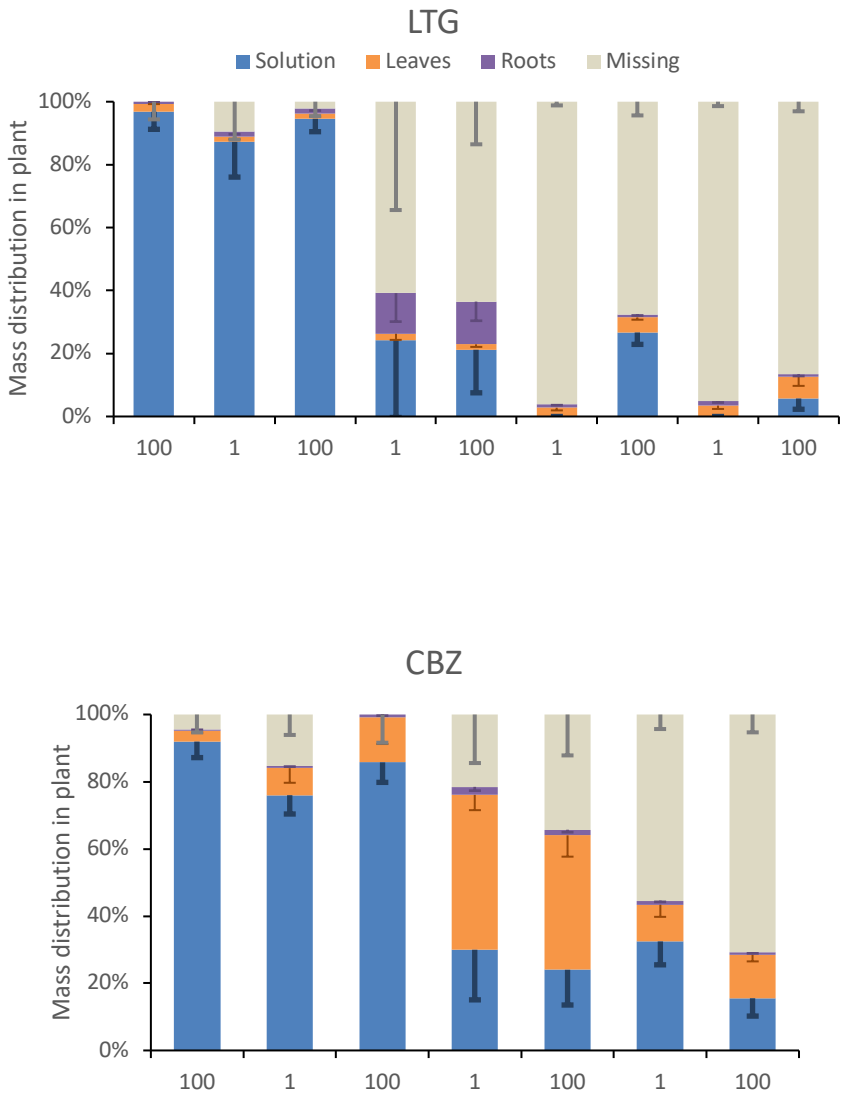

Figure 2. Mass distribution of (A) lamotrigine (LTG) and (B) carbamazepine (CBZ) at the end of the 7 day exposure period. Fraction denoted "missing" was not detected in plants or nutrient solution. Numbers on the *x*-axis represent exposure concentrations of 1 µg·L^{-1} and 100 µg·L^{-1}. Mixture and single compound exposure data were not significantly different and are therefore combined unless otherwise noted. Error bars show one standard deviation ($n \geq 3$).

Table 1. Maximum scores for plant protein alignment with human drug metabolizing CYP450 enzymes found using NCBI BLAST searches.*

Plant Protein	Human CYP	Species *A. thaliana*	Spinach	Tomato	Cucumber	CBZ
CYP1A1	CYP1A1			321		X
	CYP1A2			289		Ind
predicted CYP450 736A12-like	CYP1A1			198	214	X
	CYP1A2			199	199	Ind
CYP450 75B1	CYP1A1	212				X
	CYP1A2	199				Ind
CYP450 81F2	CYP1A1	210				X
	CYP1A2	191				Ind
predicted CYP450 93A2-like	CYP1A1			206	208	X
	CYP1A2			192	197	Ind
CYP450 703A2	CYP1A1	185	196	208	187	X
	CYP1A2	192	184	199	179	Ind
putative flavenoid 3'5' hydroxylase	CYP1A1			207		X
	CYP1A2			194		Ind
predicted CYP450 71A1-like	CYP1A1		176	206	197	X
	CYP2B6			164	161	S
	CYP2C8			179	160	S, Ind
	CYP2C9			176	176	Ind
	CYP2C19			171	172	S, Ind
	CYP2E1			179	168	X
predicted CYP450 83B1-like	CYP1A1	180	167	203		X
	CYP2B6	160		169		Ind
predicted CYP450 711A1-like	CYP3A4	179	196	196	201	S, Ind
	CYP3A5	177	191	185	185	S, Ind
	CYP3A7	171	174	177	178	S

* In the CBZ column, S indicates that CBZ is a substrate of the human CYP450, Ind indicates that CBZ is an inducer of the human CYP450, and X indicates no CBZ interaction with the human CYP450. Scores are color coded with darker colors indicating higher scores. Enzyme list was determined by finding all matches to human enzymes with scores above 200, and recording additional homologues with scores above 160.

Effects of Solution pH on PPCP Accumulation and Transformation

The UW and HUJI groups both investigated the impact of solution pH on the accumulation and metabolism of CBZ and LTG.

Effects of Rhizosphere pH. The phytoavailability of PPCPs with pK_a values between 4 and 9 may be affected by the pH of the rhizosphere (the water and soil within 2-3 mm of the root surface). Plants can alter rhizosphere pH by 2 to 3 units in either direction in response to nutrient availability; they must maintain electrochemical equilibrium as they take in nutrients. Energy for uptake of cations such as ammonium is provided via counter-transport of H^+ out of root cells, resulting in a decrease of rhizosphere pH. Uptake of anions like nitrate is accompanied by co-transport of H^+ into root cells, increasing rhizosphere pH.[2] Most plants modulate rhizosphere pH in response to the form of nitrogen available, as nitrogen accounts for up to 80% of ions taken in by plants.[3] The effects of nutrient availability and rhizosphere pH on plant accumulation of ionizable organic contaminants has not been previously

considered. In this study, we examine the effects of plant-driven rhizosphere pH changes on trends in wheat accumulation of both an ionizable and a non-ionizable contaminant to discriminate between effects on accumulation due to changes in rhizosphere pH and those due to other changes to the plants caused by differences in nutrient availability. We cultivated durum wheat with different forms of nitrogen in a growth system designed to isolate the rhizosphere (**Fig. 4**). We measured pH and pharmaceutical concentrations in the rhizosphere and related them to the phytoaccumulation of the compounds (**Figs. 5 and 6**). We found that plant-driven changes in rhizosphere pH alter accumulation of LTG (pK_a of conjugate base = 5.7), but do not affect accumulation of CBZ, a non-ionizable contaminant (**Fig. 6**). When plants received nutrient solution containing only nitrate, rather than both nitrate and ammonium, the rhizosphere pH was 1.5-2.5 units higher (**Fig. 5**). The amount of neutral LTG available in pore water correlated strongly with accumulation in roots and above-ground tissues (**Fig. 7**). Future studies on plant uptake of PPCPs should consider rhizosphere pH separately from bulk soil pH and report concentrations of nutrients available to the plants.

A manuscript describing this work has been submitted to *Environmental Science and Technology Letters*.

Effect of pH in Hydroponic Studies. The effect of solution pH on PPCPs uptake was tested for the nonionic compound CBZ and for LTG, which is positively charged at pH 4.5 (**Fig. 8**). Consistent with the results from the rhizosphere pH modulation experiments described above, solution pH did not affect CBZ uptake. In contrast, LTG uptake depended strongly on solution pH. When the pH surrounding the root was below the pK_a of LTG, uptake was hindered relative to higher pH values because the compound was present predominately as the cationic species and could bind to the negatively charged cell walls, and its permeability through the root membranes was reduced relative to the neutral species. Although uptake of LTG was related to solution pH, translocation from root to shoot was unaffected by solution pH.

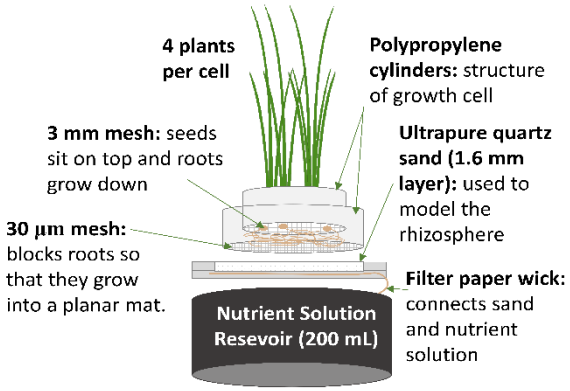

Figure 4. Exploded view of a full rhizosphere setup. Growth cells were composed of two polypropylene cylinders (i.d. 25 mm and 35 mm) with 3 mm polypropylene mesh stretched across the bottom of the smaller and 30 µm nylon mesh attached to the bottom of the larger. The smaller cylinder was glued inside the larger to leave a 3.5 mm gap between the coarse and fine meshes. Seeds were placed on top of the coarse mesh such that the roots grew down to form a planar mat on top of the fine mesh. On day 22 post-hydration, growth cells were placed into full rhizosphere setups with a 1.6 mm layer of sand to represent the rhizosphere. A strip of cellulose filter paper placed under the layer of sand connected the sand layer to the nutrient solution reservoir. Solution was replenished every 2 days. Nutrient solution was amended with lamotrigine or carbamazepine for a final concentration of 100 $\mu g \cdot L^{-1}$.

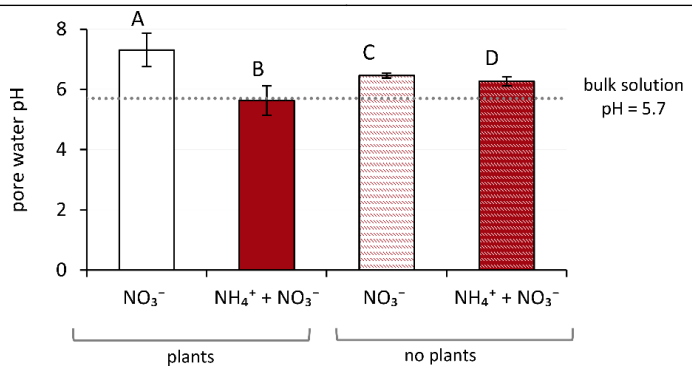

Figure 5. Wheat plants altered pore water pH in model rhizospheres in response to the form of nitrogen provided, raising pore water pH when supplied with nitrate as the sole nitrogen source. The dotted line indicates the pH of the initial nutrient solution. Letters indicate statistical significance based on an ANOVA with Welch's correction and Games-Howell *post hoc* analysis ($p < 0.05$). Bars represent mean values; error bars indicate one standard deviation ($n = 21$ for treatments with plants; $n = 9$ for treatments lacking plants).

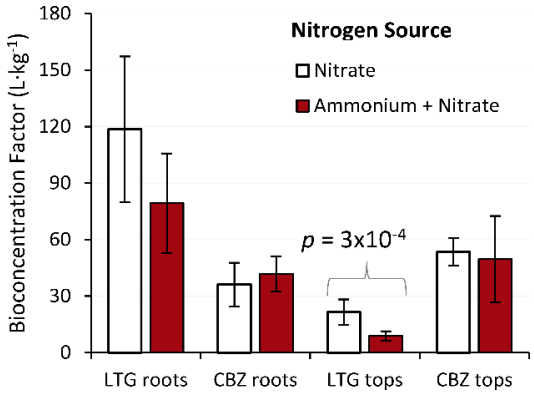

Figure 6. Bioconcentration lamotrigine (LTG) and carbamazepine (CBZ) in the roots and leaves of wheat plants supplied with the indicated nitrogen sources. The amount of LTG accumulating in the leaves of plants provided with only nitrate exceeded that in plants receiving both ammonium and nitrate by a factor exceeding two. Bioconcentration factors represent the ratio of the concentration in the plant tissue to that in the pore water. Error bars indicate one standard deviation. Concentrations of LTG, CBZ in pore water did not vary between nutrient treatments.

In addition to the effect on LTG uptake, the concentration of EP-CBZ in the roots and shoots was significantly higher at low pH (**Fig. 9**), indicating a possible effect of the presence of LTG may have on the *in planta* metabolism of CBZ. Since both compounds were taken up at relatively high and similar extents, one possible explanation for this observation that they compete for the same CYP 450 enzymes, reducing the degree of CBZ metabolism in the presence of higher concentration of LTG at the higher pH.

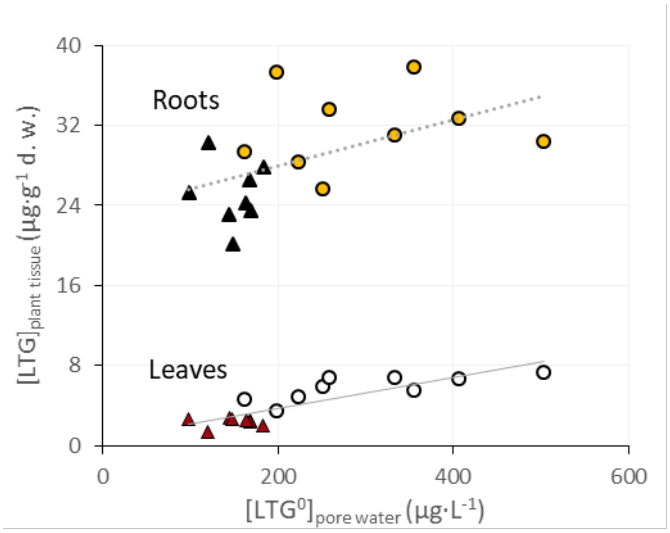

Figure 7. Lamotrigine (LTG) accumulation in wheat leaves and roots correlated with the concentration of the neutral LTG species in pore water. White and yellow circles correspond to plants provided with nitrate as the sole nitrogen source. Red and black triangles correspond to plants provided with ammonium + nitrate. Coefficients of determination (R^2) for the regressions of LTG concentrations in roots and leaves against neutral LTG concentrations in pore water were 0.73 and 0.27, respectively.

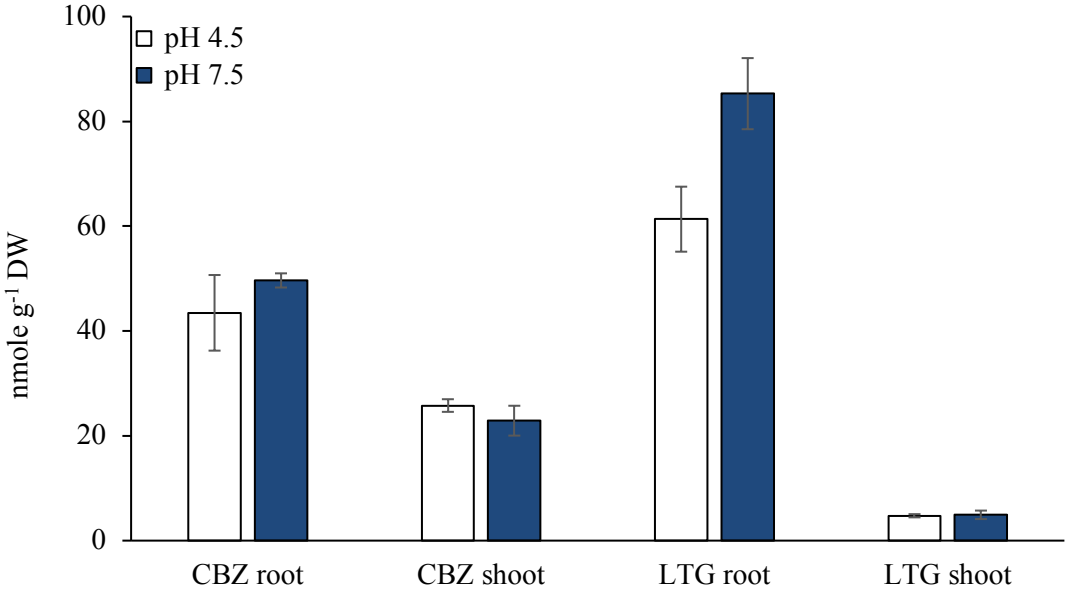

Figure 8. Measured concentration (nmol g^{-1}) of carbamazepine (CBZ) and lamotrigine (LTG) in the roots and shoots during the exposure period under external pH levels of 4.5 and 7.5. Bars represent standard errors (n = 3).

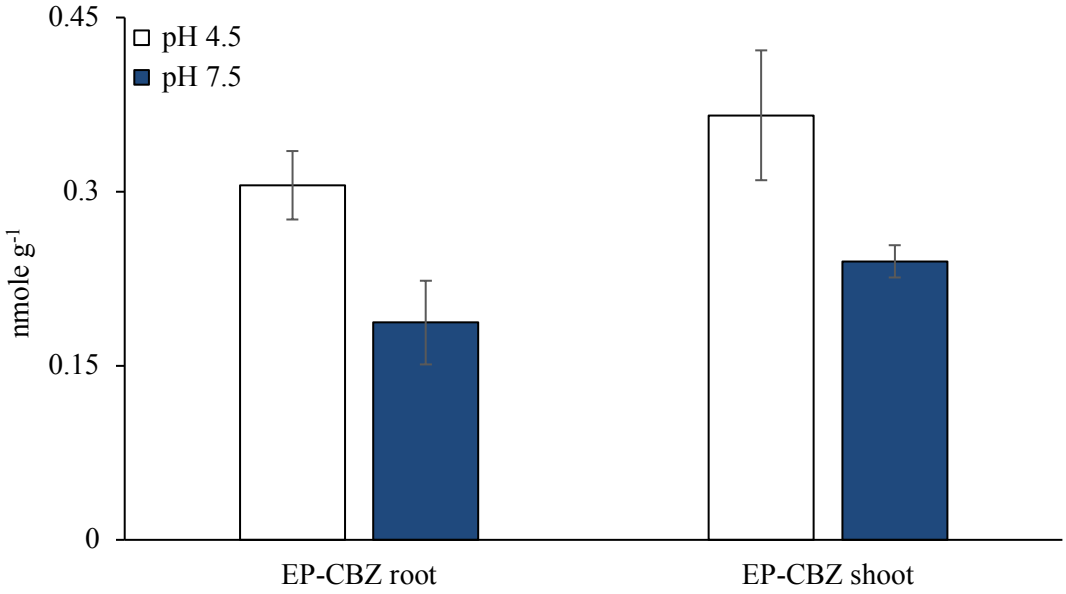

Figure 9. Measured concentration (nmol g^{-1}) of 10,11-dihydro-10,11-epoxy-carbamazepine (EP-CBZ) in the roots and shoots during the exposure period under two different external pH levels: 4.5 and 7.5. Data presented for dry weight. Bars represent standard errors (*n* = 3).

University of Wisconsin Group – Joel Pedersen, K.G. Karthikeyan, and Curtis Hedman

The group at University of Wisconsin – Madison focused on plant-driven processes impacting the accumulation and metabolism of pharmaceuticals and personal care product ingredients (PPCPs) using durum wheat (*Triticum durum*), spinach, and the model plant *Arabidopsis thaliana*. We examined the influence of plant-induced changes to rhizosphere pH on accumulation of carbamazepine (CBZ) and lamotrigine (LTG) (described above), the impact of root exudates on LTG availability in soils, the effects of exposure to PPCP mixtures on accumulation and *in planta* metabolism, the influence of transpiration on PPCP accumulation, and transcriptomic response of *A. thaliana* to exposure to CBZ.

Effects of Root Exudates on Contaminant Availability

Plants not only take in or release H^+ in response to nutrient availability, they also release a complex mixture of organic compounds. The amount and composition of plant root exudates depends on many factors, including species,[4] plant growth stage and associated bacteria,[5] water stress,[6] and nutrient deficiency.[6,7] Root exudates mixtures are known to include many common organic and amino acids, carbohydrates, and phytosiderophores. However, little is known about how changes in nutrient availability can alter the composition of root exudates or how exudates may impact sorption of PPCCs, thereby impacting PPCP availability to plants. We cultivated durum wheat hydroponically in nutrient solutions containing different ratios of nitrate to ammonia, and collected the resulting root exudates. We determining the chemical composition of the organic molecules in the root exudates by Fourier Transform - Ion Cyclotron Resonance Mass Spectrometry. We have examined the sorption of LTG to three field soils from the U.S. (**Table 2**) in the absence and presence of the different root exudates. For root exudates from plants provided only nitrate, soil organic carbon was the major driver of lamotrigine sorption, and root exudates altered sorption in the soil with low organic carbon (**Fig. 10**).

Table 2. Properties of three soil types used to test the effect of root exudates on sorption.

Analysis Method		Richford Sand	Elliot Silty Clay Loam	Bluestem Sandy Clay Loam
Size Fractionation	% Sand	87	7	50
	% Silt	6	62	31
	% Clay	7	31	27
Dry Combustion	f_{oc}	0.007	0.029	0.0042
N_2 Adsorption (BET)	Specific Surface Area ($m^2 \cdot g^{-1}$)	1.47	10	10.3
X-Ray Diffraction		Amount of clay fraction (%)		
	Quartz	85	71	67
	K-feldspar	5.6	3.6	6.9
	Plagioclase	7.9	8.6	15
	Amphibole	-	2.4	2.5
	Calcite	-	0.3	0.4
	Dolomite	-	1.8	-
	Hematite	-	-	1.2
	Mixed-layer Illite/Smectite	0	14	4.4
	Illite + Mica	0.4	8.7	1.3
	Kaolinite	0.7	1.7	1.7
	Chlorite	0.4	0.4	0.2

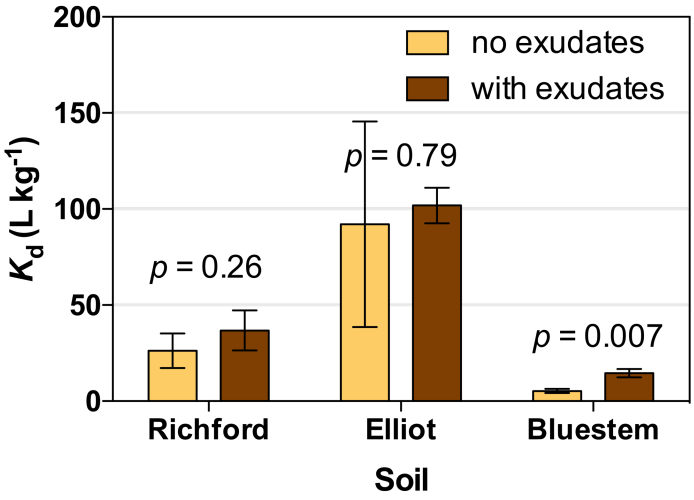

Figure 10. Calculated soil-water distribution coefficients (K_d) values of equilibration experiments between three soils (**Table 2**), lamotrigine and a 0:6 ratio of ammonium to nitrate nutrient solution in the presence or absence of root exudates. Samples without root exudates present are depicted in blue (- root exudates), samples with root exudates are shown in orange (+ root exudates). Welch's *t*-test was used for pairwise comparison between treatments, and resulting p-values are given above each soil type. High K_d values indicate high levels of lamotrigine sorption to soil. Error bars indicate one standard deviation (n = 3).

Effects of Transpiration and Binary Mixtures on PPCP Accumulation by Spinach

Previous research has shown that pharmaceuticals can accumulate in plants irrigated with reclaimed wastewater under field conditions,[13] but plant driven-processes that control differences in accumulation between compounds are not yet well understood. We tested the effects of binary compound mixtures and transpiration on spinach accumulation and

metabolism of four pharmaceuticals commonly found in treated wastewater effluent. The objectives of this study were to investigate the effects of binary mixtures on pharmaceutical accumulation and metabolism and to evaluate the relationship between transpiration and compound accumulation in spinach plants. We used a mass-balance approach to determine the relationship between plant uptake of water and pharmaceuticals, the fraction transformed within the experimental system, and whether metabolism takes place in solution or in the plant. The compounds we used were carbamazepine (CBZ), lamotrigine (LTG), amitriptyline (AMI), and fluoxetine (FLX). We selected these compounds because they have been detected in treated wastewater,[8,9] and have shown accumulation in plants in previous studies.[10–13] Additionally, CBZ induces the enzymes responsible for metabolizing LTG, AMI, and FLX in mammalian systems.[14] We found that accumulation and tissue distribution within spinach plants varied among compounds. Amitriptyline exhibited the highest overall accumulation, CBZ had the highest concentration in leaves, and LTG and FLX remained mainly in the roots (**Fig. 11**). The presence of CBZ did not affect plant accumulation of AMI, FLX, and LTG (*t*-tests, $p > 0.05$). Likewise, CBZ accumulation was not affected by the presence of the other compounds (*t*-tests, $p > 0.05$). We found that fluoxetine and amitriptyline decreased the accumulation of the primary and potentially toxic metabolite of carbamazepine, 10,11-epoxycarbamazepine (**Fig. 12**). Plant metabolism of LTG, AMI, and FLX has not been previously reported to our knowledge. We estimated the extent of metabolism for all four compounds using a mass balance approach. For CBZ and LTG, we did not detect any compound loss for mixture or single compound treatments, i.e. all of the compound initially added to the nutrient solution was detected in either the plant or the nutrient solution at the end of the exposure period, within the standard deviations of the measurements (**Fig. 13**). Compound accumulation in spinach plants strongly correlated with accumulation predicted by simply considering the transpiration volume and exposure concentrations, although the ratio between transpiration-based and actual accumulation varied among tested compounds (**Fig. 14**). Amitriptyline and fluoxetine have similar physico-chemical properties, but they exhibited different trends in uptake (**Fig. 15**). We hypothesize that transporters in root cell membranes impact compound transport into the plant and cause some of the observed differences among compounds (**Fig. 16**). Our findings highlight the need to consider plant physiology and mixture effects in studying the accumulation of polar and ionizable organic contaminants and their metabolites. A manuscript of this work has been drafted and will be submitted soon.

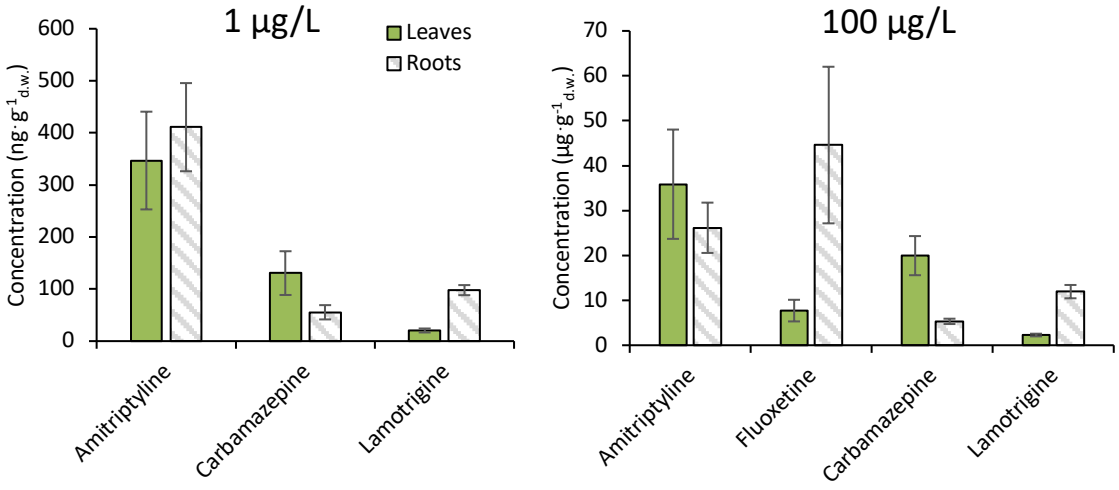

Figure 11. Concentrations of parent compounds in leaves and roots of spinach plants exposed to (A) 1 µg·L^{-1} or (B) 100 µg·L^{-1} of the indicated compounds. Data from mixture and single compound treatments are combined, as no significant differences were found. Error bars represent one standard deviation ($n \geq 7$).

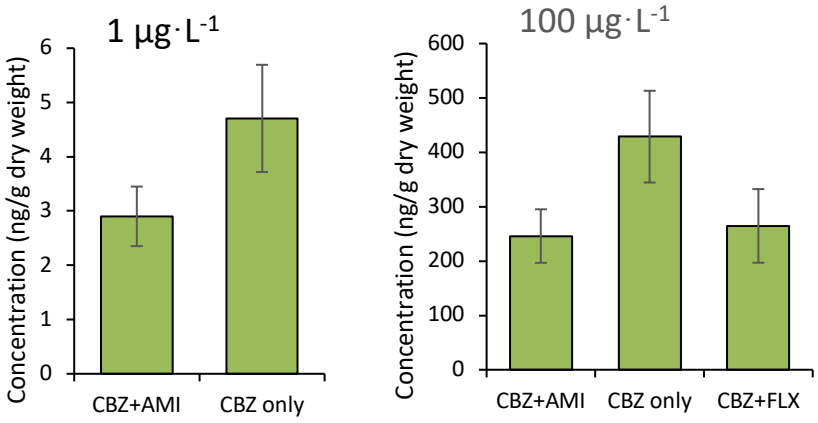

Figure 12. Accumulation of 10,11-epoxycarbamazepine in spinach leaves is lower when plants are exposed to carbamazepine (CBZ) alongside amitriptyline (AMI) or fluoxetine (FLX) at the 1 µg·L^{-1} (A) and 100 µg·L^{-1} (B) exposure levels ($p < 0.05$, Dunnett's tests). Error bars represent one standard deviation ($n \geq 4$).

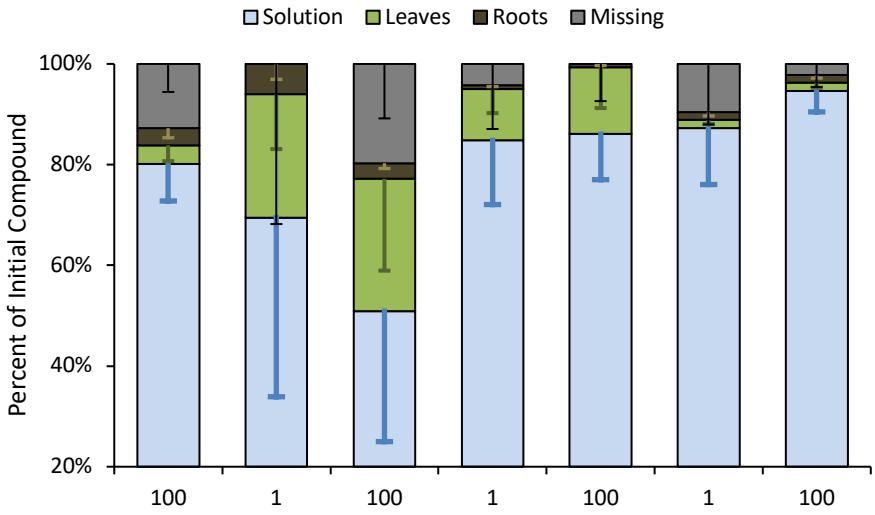

Figure 13. Mass balance for compounds taken up by spinach plants exposed for 7 days. The "missing" fraction denotes the difference between the initial amount of compound added to nutrient solution and the amount detected in nutrient solution and plants and the end of the exposure period. We found measureable loss of amitriptyline (AMI) and fluoxetine (FLX) in the 100 µg·L^{-1} exposure, but not for carbamazepine (CBZ) or lamotrigine (LTG) at either exposure concentration or AMI at 1 µg·L^{-1}. Each bar includes mixture and single compound treatments. CBZ data includes measured CBZ metabolites. Error bars represent one standard deviation ($n > 7$).

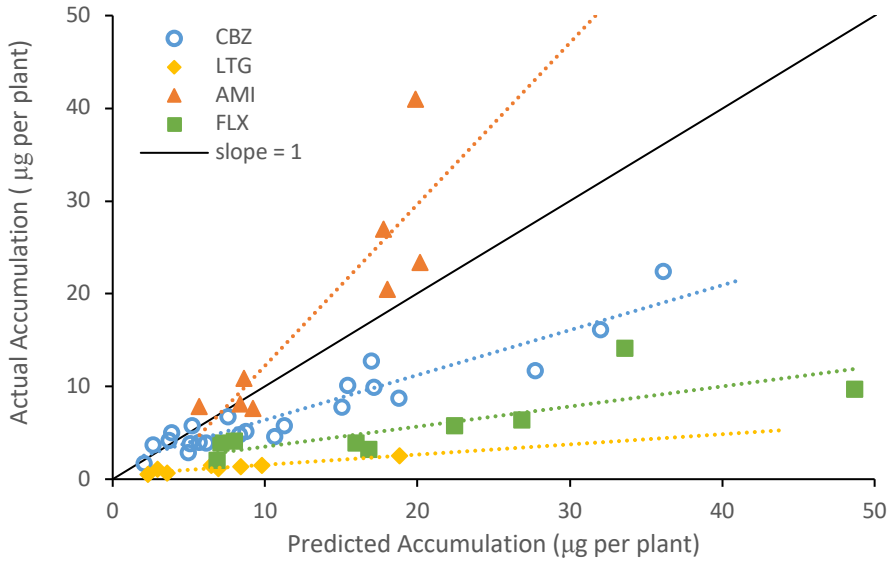

Figure 14. Predicted and actual accumulation for plants exposed to 100 µg·L^{-1} of carbamazepine (CBZ), lamotrigine (LTG), amitriptyline (AMI), and/or fluoxetine (FLX). Predicted accumulation correlates with actual accumulation ($p \leq 0.01$), but the correlation slope varies considerably among compounds and none of them are close to one (black line). Predicted accumulation is based on the concentration of the compound in the water and the volume of water transpired by the plant. Actual accumulation is the total mass of compound measured in plant roots and leaves.

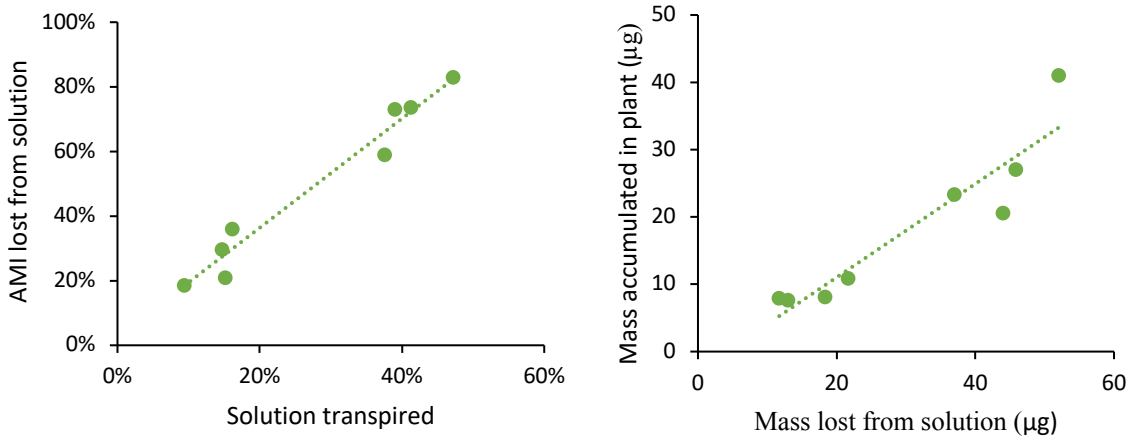

Figure 15. Amitriptyline (AMI) loss from solution correlated with (A) transpiration and (B) accumulation in the plant ($p < 0.001$). Strong correlation between transpiration and loss from solution indicates that uptake into the plant is a dominant mechanism for loss from solution. A larger fraction of AMI was lost from solution relative to the fraction of water transpired, indicating mechanisms beyond transpiration are important for AMI uptake. However, more AMI mass was lost from solution than accumulated in the plant, indicating AMI degradation in the plant.

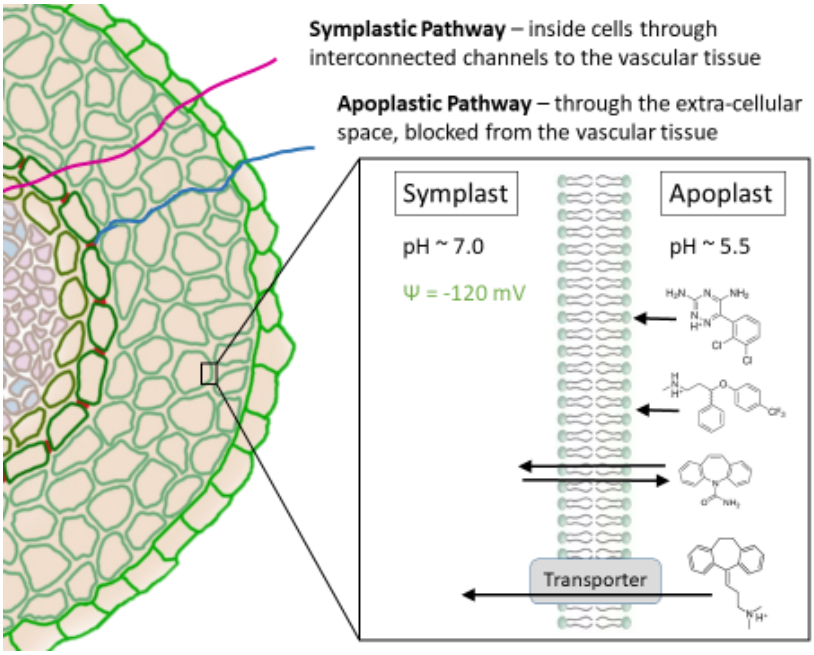

Figure 16. Schematic showing the symplastic and apoplastic pathways for a molecule to reach the vascular tissue of a plant root. Molecules that cross a cell membrane and move between cells through interconneted channels (symplastic pathway) are able to reach the vascular tissue in the center of the root and be transported with the transpiration stream to the leaves. Molecules that diffuse into the intercellular space but do not cross into root cells (apoplastic pathway) are blocked from entering the vasuclar tissue by the Casparian strip, a waxy barrier. Neutral molecules can diffuse across cell membranes to travel symplastically. Charged molecules diffuse very slowly across cell membranes. However, root cells have negative membrane potential, which drives accumulation of cations when they are not blocked by the cell membrane. Transporter proteins in root cells may facilitate the corssing of the cell membrane for some compounds.

Effect of Carbamazepine on Gene Expression in *Arabidopsis thaliana*

Plants under stress alter functions that may affect accumulation of xenobiotic compounds, including uptake of water, ion balance and transport, and nutrient breakdown and allocation.[15] Crops are exposed to mixtures of wastewater-derived contaminants, and transcriptional responses to one contaminant (e.g., carbamazepine) may impact the accumulation of other compounds. Other pharmaceuticals may cause similar responses to that of carbamazepine. The objective of this study was to determine how exposure to CBZ alters gene expression in *A. thaliana*. *A. thaliana* is a well-studied model brassica (closely related to cabbage, turnips, and broccoli) with a short life cycle and fully sequenced genome. An advantage of working with *A. thaliana* is the availability of a wide range of genetic tools including mutants and gene expression arrays, both of which we have found useful for studying interactions between plants and pharmaceuticals. We hypothesized that enzymes analogous to those up-regulated by CBZ in humans are also up-regulated by CBZ in plants. To test this hypothesis, we exposed *A. thaliana* to CBZ (0, 1, and 100 $\mu g \cdot L^{-1}$ CBZ for 24 h) and examined the response of the entire transcriptome using Affymetrix GeneChips. Additional plants were grown and exposed to CBZ at the same time as the plants used for transcriptome analysis to verify the accumulation of CBZ in aerial tissues. Plants from both treatments accumulated detectable levels of CBZ, but the two main metabolites of CBZ were detectable only in the high treatment (**Fig. 17**). However, no definitive differential expression was detected between treatments (**Fig. 18**), likely due to the amount of variation in control

samples. In addition to the effect of biological variability, the effect of CBZ on the *A. thaliana* transcriptome may be ambiguous at the whole-plant level. Although there were no significantly up- or down-regulated genes, the data are suggestive of gene expression changes that could take place earlier or later than the tested exposure time or with exposure to higher concentrations, as the environmentally relevant (1 $\mu g \cdot L^{-1}$) and high (100 $\mu g \cdot L^{-1}$) CBZ treatments produced different expression profiles (**Fig. 18**). Future gene expression experiments should examine different time points during the exposure. There may be benefit of using plant cell culture instead of whole plants to decrease inter-replicate variation.

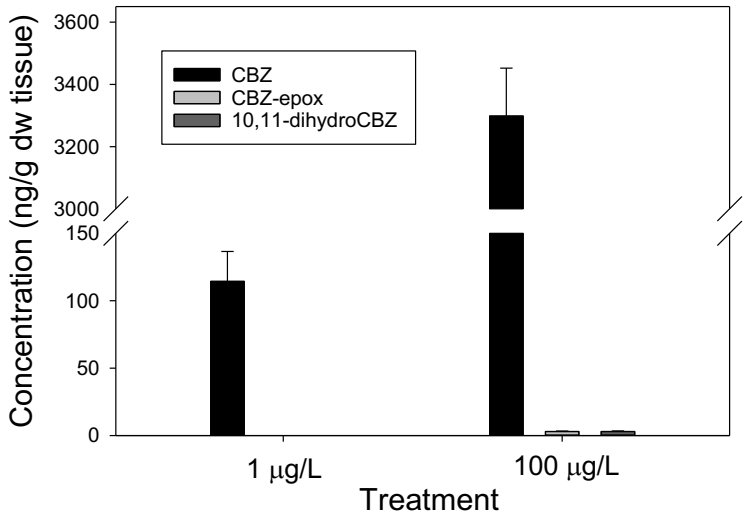

Figure 17. Extracts of leaf tissue from the low (1 $\mu g \cdot L^{-1}$) and high (100 $\mu g \cdot L^{-1}$) treatments had detectable levels of carbamazepine (CBZ). The two main metabolites of CBZ were detectable only in the high treatment. Nutrient solution concentrations did not change significantly between the start and end of the exposure period (data not shown). The metabolites were detectable only in the high treatment, but at levels below limits of quantitation (LOQs).

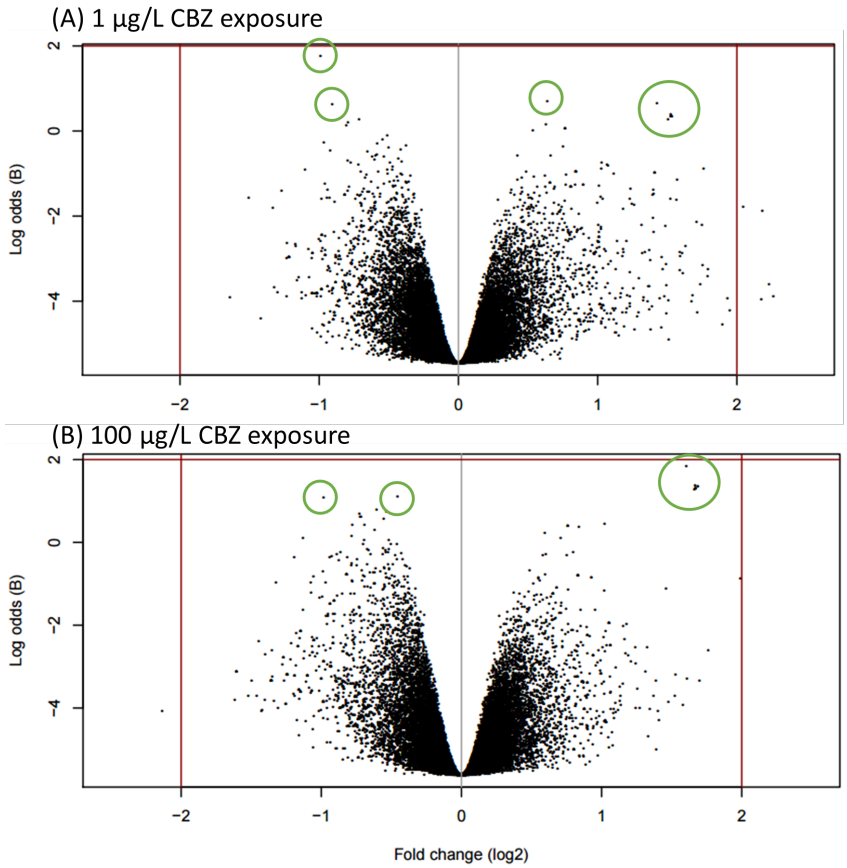

Figure 18. Volcano plots for (A) low CBZ and (B) high CBZ show fold change from control vs. odds of differential expression. Each point represents a gene transcript. The red lines indicate minimum values for gene expression to be considered significantly different from controls. Genes with the highest differential expression are circled in green.

HUJI group – Moshe Shenker, Benny Chefetz, and Jorge Tarchitzky

The HUJI group focused on the effect of PPCP chemical characteristics and soil and water properties on the uptake, translocation and redistribution of selected pharmaceuticals and personal care product ingredients (PPCPs) and their metabolites within plants. As representative plants, we used tomato, cucumber, and lettuce (*Lactuca sativa*).

Two experimental approaches were taken. In the first approach, we used a wide range of greenhouse experiments to screen for combination effects of different PPCPs varying in their physicochemical properties (**Table 3**), environmental characteristics such as soil properties (**Table 4**) and water quality (**Table 5**), and crop type on uptake. These experiments aimed to examine translocation to above ground edible commercial size plant parts (cucumber and tomato fruits, lettuce leaves). In the second approach, hydroponic experiments with cucumber as a model plant were conducted to elucidate the mechanisms behind the obtained results. These experiments focused on the uptake of the antiepileptic drugs carbamazepine (CBZ) and lamotrigine (LTG), both detected at relatively high concentration in treated wastewater (TWW) and found to accumulate at relatively high concentrations in plants.

Table 3. Selected physicochemical properties and therapeutic uses of the studied PPCPs

Name	Formula	MW (g mol^{-1})	log K_{ow}	pK_a	C_w^{sat} [a] (mg L^{-1})	Therapeutic use
Bezafibrate	$C_{19}H_{20}ClNO_4$	361.83	4.25 [16]	3.6 [17]	5 [18]	Hyperlipidemia
Caffeine	$C_8H_{10}N_4O_2$	194.19	-0.07 [16]	---	2.06×10^4 [16]	Stimulant
Carbamazepine	$C_{15}H_{12}N_2O$	236.27	2.45 [16]	---	125 [19]	Anticonvulsant
Clofibric acid	$C_{10}H_{11}ClO_3$	214.65	2.57 [16]	3.2 [17]	582.5 [20]	Hyperlipidemia
Gemfibrozil	$C_{15}H_{22}O_3$	250.34	4.77 [16]	4.7 [21]	3.5×10^4 [22]	Hyperlipidemia
Ibuprofen	$C_{13}H_{19}O_2$	206.29	3.97 [16]	4.91 [23]	41[16]	Anti-inflammatory
Ketoprofen	$C_{16}H_{14}O_3$	254.29	3.12 [16]	4.45 [23]	268 [16]	Anti-inflammatory
Lamotrigine	$C_9H_7Cl_2N_5$	256.10	2.57 [16]	5.34 [24]	186 [25]	Anticonvulsant
Metoprolol	$C_{15}H_{25}NO_3$	267.36	1.88 [16]	9.7 [26]	1.4×10^4 [22]	β blocker
Naproxen	$C_{14}H_{14}O_3$	230.27	3.18 [16]	4.45 [21]	2.04×10^4 [21]	Anti-inflammatory
Sildenafil	$C_{22}H_{30}N_6O_4S$	474.59	2.75 [16]	6.4, 7.4 [b]	3200 [27]	Vasoactive
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	253.28	0.89 [16]	1.80, 5.60 [28]	380 [16]	Antibacterial
Sulfapyridine	$C_{11}H_{11}N_3O_2S$	249.29	0.35 [16]	2.30, 8.40 [28]	268 [16]	Antibacterial
EP-CBZ	$C_{15}H_{12}N_2O_2$	252.27	1.97 [29]	–0.9, 14.8 [30]	---	Carbamazepine metabolites
DiOH-CBZ	$C_{15}H_{14}N_2O_3$	270.28	0.81 [29]	–1.5, 11.7, 12.3, 14.0 [30]	---	
2-OH-CBZ	$C_{15}H_{12}N_2O_2$	252.27	2.66 [31]	9.30 [31]	---	
3-OH-CBZ	$C_{15}H_{12}N_2O_2$	252.27	2.66 [31]	9.46 [31]	---	

[a] Aqueous solubility

Table 4. Selected properties of the studied soils.

	Sandy soil	Aeolian sand	Alluvial soil
Clay content %[a]	7.50	12.5	40.0
Silt content %[a]	0	7.5	37.5
Sand content %[a]	92.5	80.0	22.5
Texture [a]	Sand	Sandy loam	Clay loam-clay
Organic matter %[b]	0.39 ± 0.02	0.73 ± 0.09	1.8 ± 0.2
Specific surface area (m^2 g^{-1})[c]	18 ± 2	28 ± 2	27 ± 4
pH[d]	7.6 ± 0.6	7.9 ± 0.3	7.9 ± 0.1
EC (dS m^{-1})[d]	0.1 ± 0.03	1.8 ± 0.2	1.3 ± 0.1

Average values ± standard deviation are presented; [a] Determined by hydrometer analysis and classified according to the International System; [b] Determined by weight loss on ignition at 400°C for 8 h; [c] Determined by the ethylene glycol monoethyl ether (EGME) method; [d] Measured in a saturated soil paste extract.

Table 5. Irrigation water characteristics and measured PPCP concentrations.[a]

	Fresh water (FW)		Treated wastewater (TWW)	
Total suspended solids (TSS; mg L^{-1})	N.D.[a]		2.0-8.8	
Biological oxygen demand (BOD; mg L^{-1})	N.D.		6.7-18.1	
Chemical oxygen demand (COD; mg L^{-1})	N.D.		27.0-45.0	
pH	7.2		7.1-7.7	
EC (dS m^{-1})	0.7		1.3-1.6	
N total (mg L^{-1})	N.D.		10.8-11.3	
P total (mg L^{-1})	N.D.		2.0-10.6	
Cl^-(mg L^{-1})	96.0		96.0-277.0	
NO_3^- (mg L^{-1})	12.0		1.9 - 8.0	
HCO_3^- (mg L^{-1})	167.0		262.0-292.0	
Na^+ (mg L^{-1})	1.4		7.3-8.8	
NH_4^+ (mg L^{-1})	N.D.		0.7-10.9	
K^+ (mg L^{-1})	<2.0		22.8-31.3	
	FW	**FW-spiked**	**TWW**	**TWW-spiked**
Bezafibrate (µg L^{-1})	N.D.	0.86 ± 0.27	0.30 ± 0.33	1.30 ± 0.66
Caffeine (µg L^{-1})	N.D.	1.31 ± 1.02	0.59 ± 0.78	1.39 ± 0.81
Carbamazepine (µg L^{-1})	N.D.	0.91 ± 0.45	1.06 ± 0.39	1.95 ± 0.51
Clofibric acid (µg L^{-1})	N.D.	1.03 ± 0.48	N.D.	1.09 ± 0.60
Gemfibrozil (µg L^{-1})	N.D.	0.87 ± 0.45	0.02 ± 0.00	0.97 ± 0.45
Ibuprofen (µg L^{-1})	N.D.	0.67 ± 0.39	0.13 ± 0.12	0.87 ± 1.02
Ketoprofen (µg L^{-1})	N.D.	0.98 ± 0.90	0.06 ± 0.00	1.70 ± 0.39
Lamotrigine (µg L^{-1})	N.D.	0.97 ± 1.02	1.49 ± 0.90	2.44 ± 1.29
Metoprolol (µg L^{-1})	N.D.	1.07 ± 0.90	0.09 ± 0.06	1.32 ± 1.02
Naproxen (µg L^{-1})	N.D.	0.85 ± 0.75	0.45 ± 0.06	1.38 ± 0.96
Sildenafil (µg L^{-1})	N.D.	0.49 ± 0.06	0.03 ± 0.00	0.92 ± 0.12
Sulfamethoxazole (µg L^{-1})	N.D.	0.79 ± 1.26	0.28 ± 0.36	0.82 ± 1.53
Sulfapyridine (µg L^{-1})	N.D.	1.00 ± 0.30	0.17 ± 0.18	0.74 ± 1.26

[a] Mean values and standard deviation are presented for pharmaceutical compounds (*n* = 9). N.D., Not detected.

Effects of Crop Type and PPCP, Soil, and Water Properties on Accumulation and Metabolism

All the studied PPCPs were detected in plants (fruits and/or leaves) in at least one soil–irrigation combination in the greenhouse experiments (**Figs. 19 and 20**). In cucumber and tomato plants, all PPCPs that were detected in the fruits were also detected in the leaves; however not all PPCPs that were detected in the leaves were detected in the fruits, suggesting the involvement of different translocation mechanisms. Large differences in accumulation of nonionic versus ionic PPCPs in the plants was found. The nonionic PPCPs that were found in the plants in this study were caffeine, CBZ, LTG and sulfapyridine. The ionic PPCPs found in the plants were the positively charged metoprolol and the negatively charged bezafibrate, clofibric acid, gemfibrozil, ibuprofen, ketoprofen, naproxen, and sulfamethoxazole. Sildenafil, also found in the plants, can change its charge from positive to neutral to negative based on pH. At the bulk soil pH ~64% of sildenafil is negatively charged, ~30% is neutral and ~5% is in the form of a zwitterion. Once crossing the root membrane, in the apoplast under a pH of ~5.5, sildenafil is mostly positively charged, while inside the root cells, under the pH of the cytoplasm (~7-7.5), it is mostly negatively charged and may therefore undergo ion trapping. The concentrations of all ionic compounds in the plant material were usually much lower than those of the nonionic, in agreement with the lower permeability of cell membranes to ionic organic compounds.

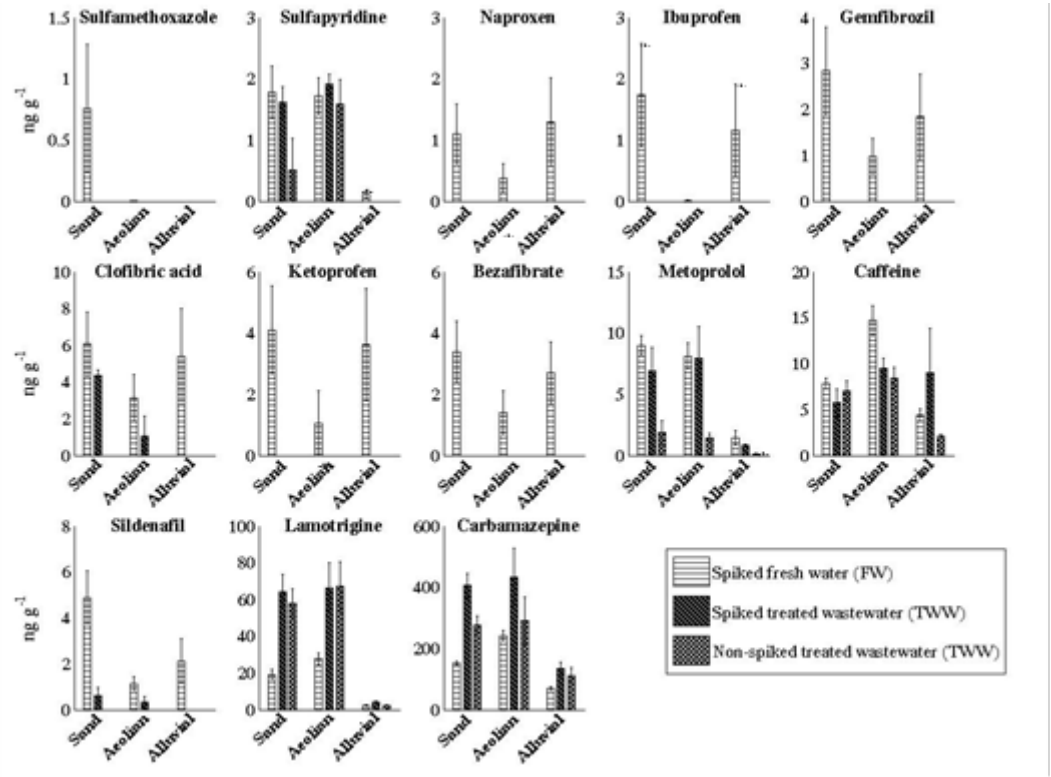

Figure 19. Concentrations of PPCPs in leaves of cucumber plants (ng g^{-1} dry weight) grown in the indicated soils and irrigated with the indicated water source. Mean concentrations and standard errors are shown (*n* = 9 for spiked FW, *n* = 4 for spiked and non-spiked TWW). * indicates concentrations between limits of detection and quantitation (LOD and LOQ).

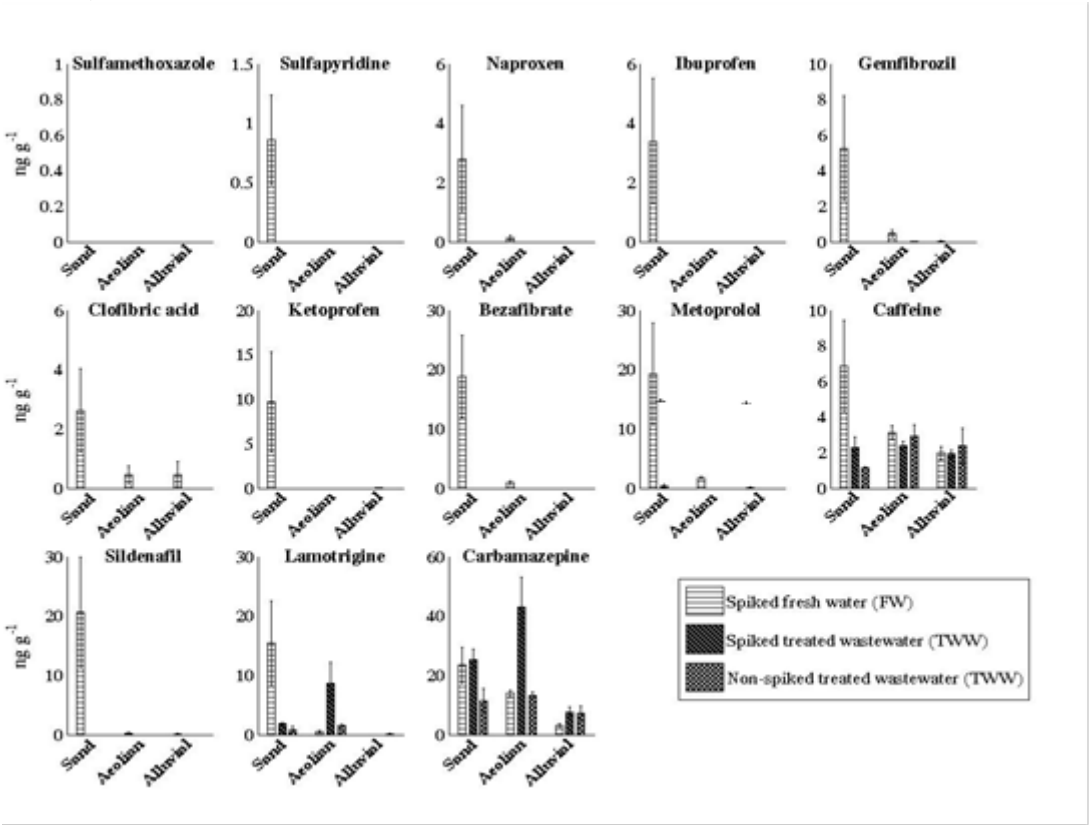

Figure 20. Concentrations of pharmaceuticals in fruit of cucumber plants (ng g^{-1} dry weight) grown in the indicated soils and irrigated with the indicated water source. Mean concentrations are shown; bars represent standard errors (*n* = 9 for spiked FW, *n* = 4 for spiked and non-spiked TWW). * indicates concentrations between LOD and LOQ.

Effect of Pharmaceutical Physicochemical Properties on Plant Uptake. We studied a large range of PPCPs to allow us to evaluate the influence of their physicochemical properties on plant uptake. The combination of PPCP physicochemical properties, soil characteristics, and plant type and possibly the growing season (summer vs winter) affected uptake and translocation of the PPCPs. The soil solution pH ranged between 7.57 and 7.94; we therefore evaluated compound hydrophobicity using log *D* calculated for pH 7.8 (**Fig. 21a**). Compounds with a higher lipophilicity (LTG and CBZ with log *D* values of 2.12 and 2.45), were taken up and translocated more than less lipophilic compounds (**Fig. 21a**). Metoprolol, which was positively charged at the experimental pH and possesses a log *D* of 0.36, was taken up and translocated at relatively high concentrations. The PPCPs with the lower lipophilicity were mostly negatively charged under the soil pH relevant for this set of experiments.

The pK_a of the compounds (**Fig. 21b**) played a major role in uptake. Negatively charged PPCPs where taken up and translocated significantly less than were the non-ionized CBZ, LTG (partly positively charged at low pH levels and neutral in the soil solution under the described experimental setup; pK_a 5.34) and metoprolol (positively charged in the soil solution under the described experimental setup; pK_a 9.70). These results point to the importance of considering not only the lipophilicity of a compound when predicting uptake; ionization of the compound has a substantial role in governing uptake into plant roots and translocation within the plant.

The combination effect of the soil properties was also evident where uptake was more pronounced in the sandy soils relative to the heavier alluvial soil. Translocation into the leaves was lower in lettuce, a result that may be attributed to the growing season. The lettuce plants were grown in winter leading to a lower transpiration rate, while the cucumber and tomato plant were grown in the summer.

Carbamazepine Metabolites in Plant Tissues

We analyzed CBZ and its major metabolites in some of the plant tissues. In cucumber and tomato leaves, the parent compound, CBZ, comprised 58% and 53%, respectively, on a molar basis, of the analyzed species (analyzed only for the spiked TWW irrigated aeolian soil). In lettuce leaves it comprised an average of 97% in all soils irrigated with spiked FW, 89% in all soils irrigated with spiked TWW and 84% in the non-spiked TWW irrigated soils, with no significant difference between soils and irrigation treatments (**Fig. 22a**). For cucumber and tomato leaves, the second dominant compound was 10,11-epoxycarbamazepine (EP-CBZ) followed by *trans*-dihydroxycarbamazepiine (*trans*-DiOH-CBZ).

In the lettuce leaves in the spiked and non-spiked TWW there was no difference between the EP-CBZ and the *trans*-DiOH-CBZ, while in the spiked FW irrigated soils the only metabolite found was EP-CBZ. Similar to the CBZ dominance in the leaves, CBZ was dominant in the cucumber fruit and *trans*-DiOH-CBZ was found at 9% followed by EP-CBZ with 6%. In tomato fruits, CBZ was found at 65% followed by *trans*-DiOH-CBZ with 29% and EP-CBZ with 5% (**Fig. 22a**). Combining the concentrations found for CBZ and its metabolites in each plant and different plant parts (**Fig. 22b**) resulted in an increase from 286 ng g^{-1} of CBZ alone to 562 ng g^{-1} of CBZ and its major metabolites in the tomato leaves and from 719 ng g^{-1} of CBZ alone to 1282 ng g^{-1} of CBZ and its major metabolites in the cucumber leaves. In lettuce leaves the change in concentration was less pronounced with a slight increase from 194 to 215 ng g^{-1}, perhaps a result of the shorter growing period during the winter month. In the fruits, these values changed from 2.8 to 4.5 ng g^{-1} for the tomato and from 55.4 to 66.8 ng g^{-1} for the cucumber. These results emphasize the need to quantify both the parent compound and its metabolites to understand PPCPs uptake, translocation and accumulation in plants.

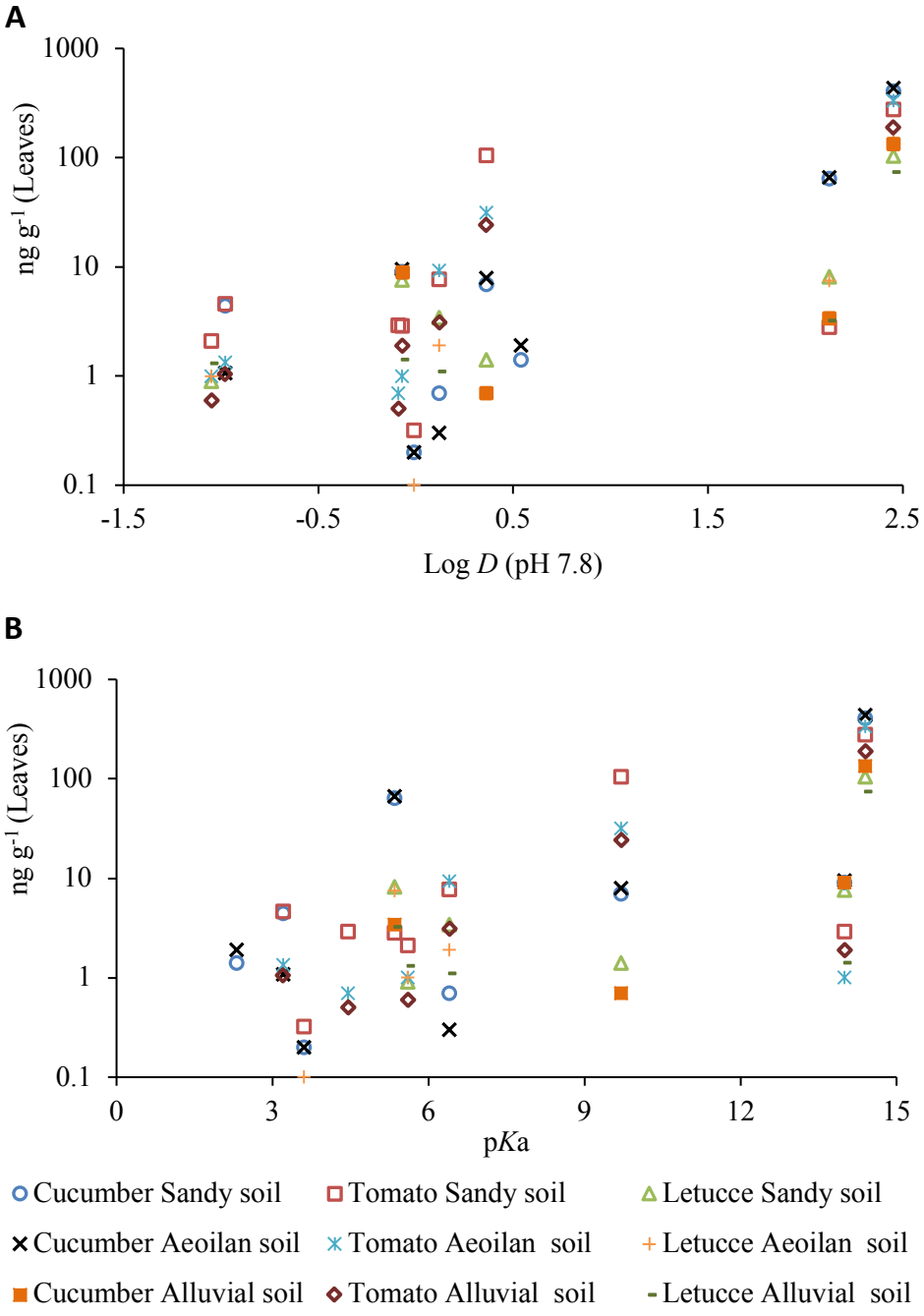

Figure 21. Relationship between the concentrations of pharmaceuticals in leaves of cucumber, tomato and lettuce plants (ng g^{-1} dry weight) irrigated with spiked treated wastewater, grown in sandy soil, aeolian soil or Alluvial soil and the and (A) the log *D* at pH 7.8 or (B) the pK_a of the pharmaceuticals.

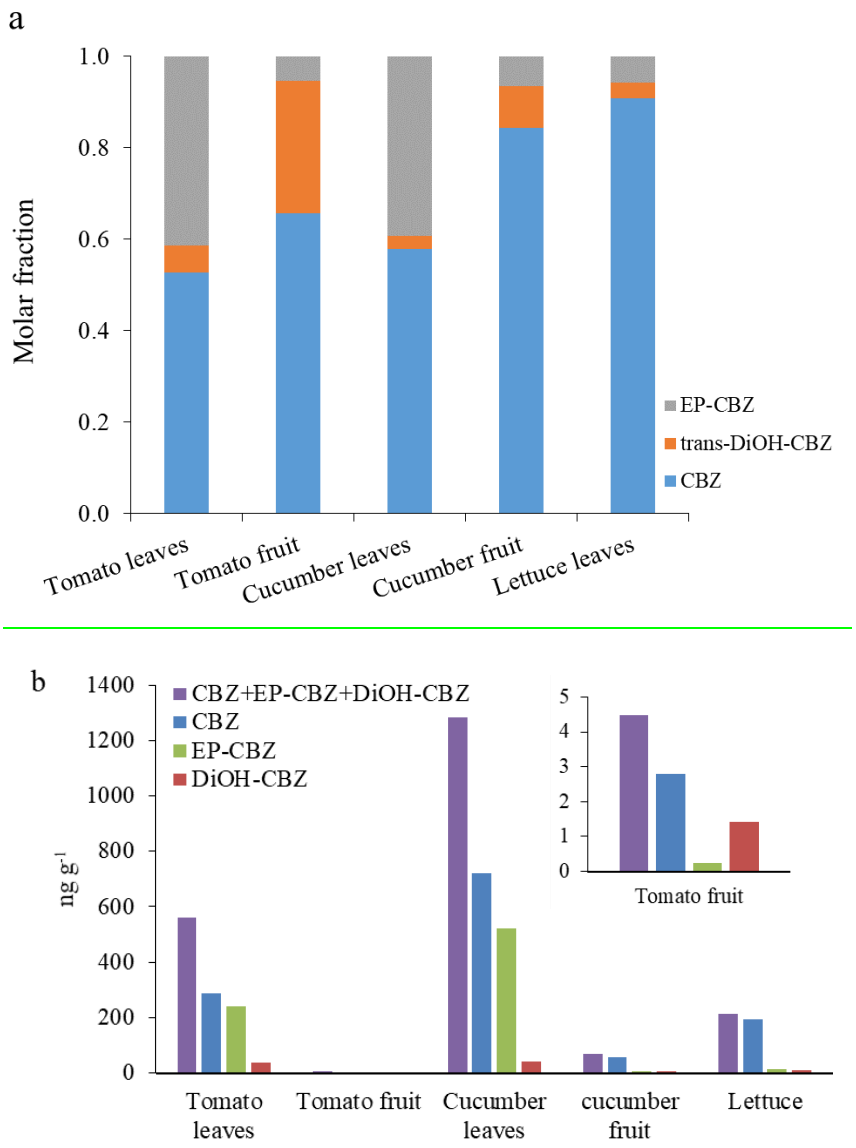

Figure 22. (a) Distribution of carbamazepine (CBZ) and its metabolites in tomato and cucumber leaves and fruits, and in lettuce leaves grown in aeolian soil irrigated with spiked treated wastewater. (b) The combined concentration of CBZ and its main metabolites (EP-CBZ and DiOH-CBZ) compared to the individual concentration of each compound in the plant material. An insert graph with expanded *Y*-axis is shown for tomato fruits. Data presented for dry weight.

Effect of DOM on Plant Uptake of PPCPs

We employed hydroponic experiments to elucidate the discrete effects of physiochemical characteristics of PPCPs and water properties on specific mechanisms involved in the uptake and translocation within plants. In this section, we discuss the effects of solution dissolved organic matter (DOM) concentration on the uptake of PPCPs. In contrast to PPCPs uptake from pure nutrient solution, which has been documented in many studies,[35,11] DOM is always present in soil solution. In this section, the effect of DOM on PPCP uptake was studied by adding DOM to the nutrient solution. Among the tested PPCPs, only CBZ and LTG were analyzed separately in the shoots and roots, while naproxen and ketoprofen were analyzed for the whole plant, root and shoot combined. Due to the lack of differences in results obtained for the different DOC levels, results are presented for 16.7 mg L^{-1} for LTG and CBZ and 8.4 mg L^{-1} for ketoprofen and naproxen.

The presence of DOM in the nutrient solution reduced CBZ and LTG uptake, with higher concentrations in the roots in the absence of DOM (**Fig. 23**). Once in the root, we observed no difference in translocation of PPCPs into the shoot. Carbamazepine has a relatively high sorption affinity to bulk DOM,[3] therefore the decreased uptake in the presence of DOM may be attributed to reducing the fraction of CBZ available for plant uptake. Lamotrigine (pK_a = 5.34) was partially positively charged in the presence of DOM (pH 5.6) and even more so in the absence of DOM (pH 4), may bind to the negatively charged moieties in the DOM further reducing its availability for plant uptake.[37] This is in addition to LTG binding to the negatively charged cell walls, thus reducing uptake into the root. Among the CBZ metabolites, only EP-CBZ was found, with no effect of DOM presence (**Fig. 24**).

Naproxen and ketoprofen possess similar physicochemical characteristics and were both found at relatively low concentrations in the whole plant, root and shoot combined. Naproxen accumulation was unaffected by the presence of DOM (**Fig. 25**). These results are supported by Maoz and Chefetz,[36] who reported naproxen to be negligibly sorbed to bulk DOM. Since the physicochemical properties of ketoprofen are similar to naproxen, the same may be assumed for ketoprofen. Both compounds are negatively charged and are therefore taken up to a smaller extent relative to nonionic or cationic organic compounds.

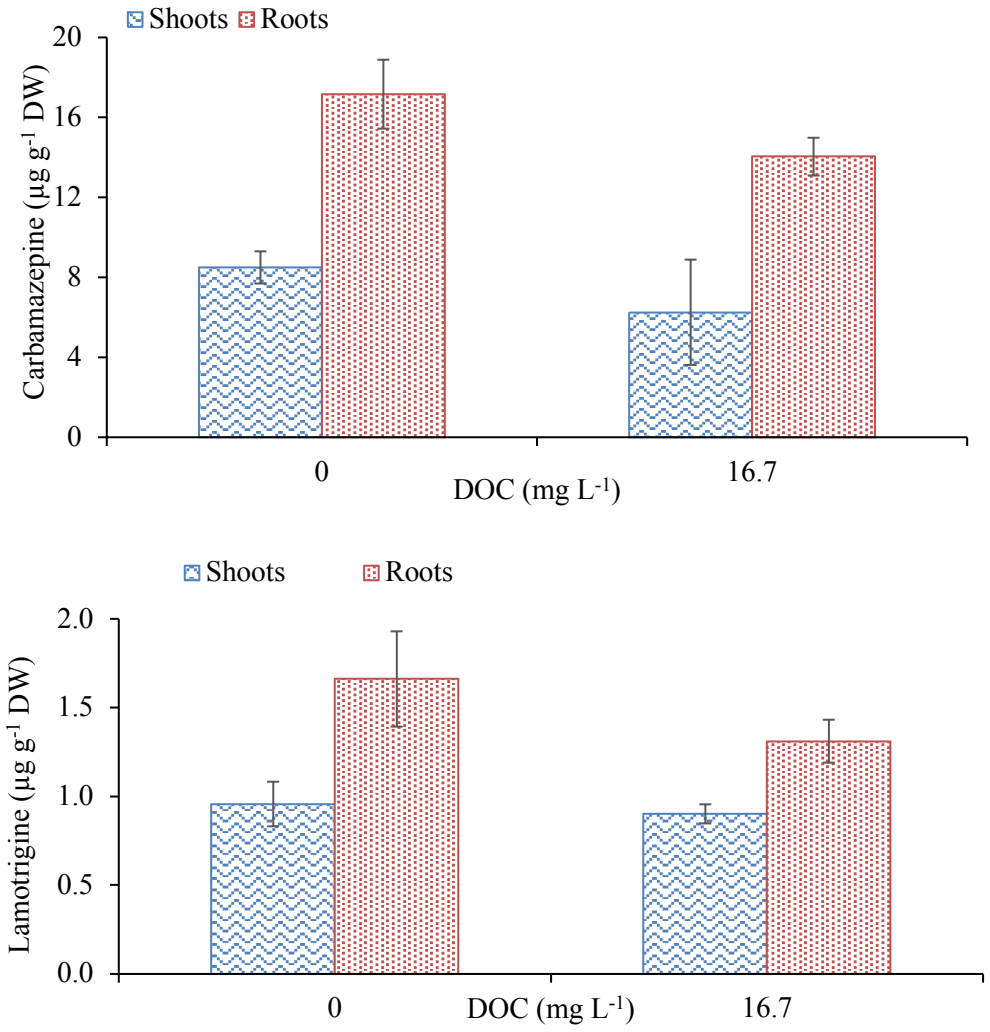

Figure 23. Carbamazepine (A) and Lamotrigine (B) measured in the cucumber shoots and root during the exposure period (48 h) in the absence (0 mg L^{-1}) and presence (16.7 mg L^{-1}) of DOM, presented as DOC concentration. Mean values are shown (n = 3); bars represent standard errors; asterisks represent significant differences.

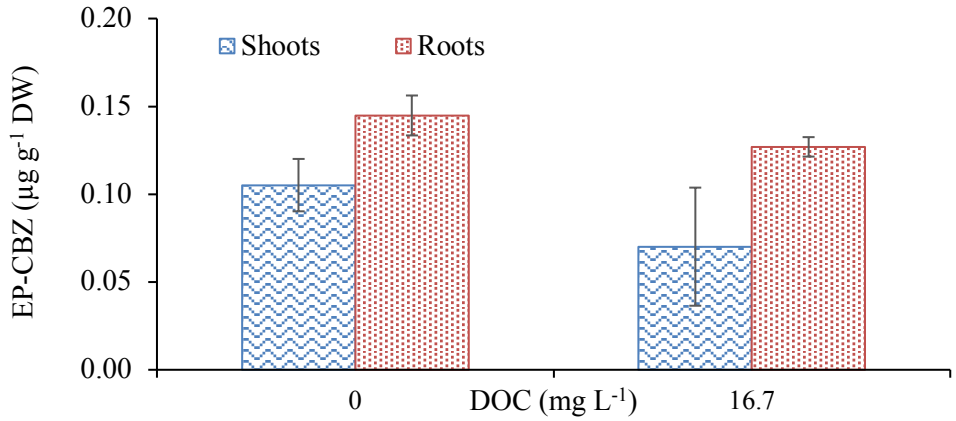

Figure 24. EP-CBZ measured in the cucumber shoots and root during the exposure period (48 h) in the absence (0 mg L^{-1}) and presence (16.7 mg L^{-1}) of DOM, presented as DOC concentration. Mean values are shown (*n* = 3); bars represent standard errors.

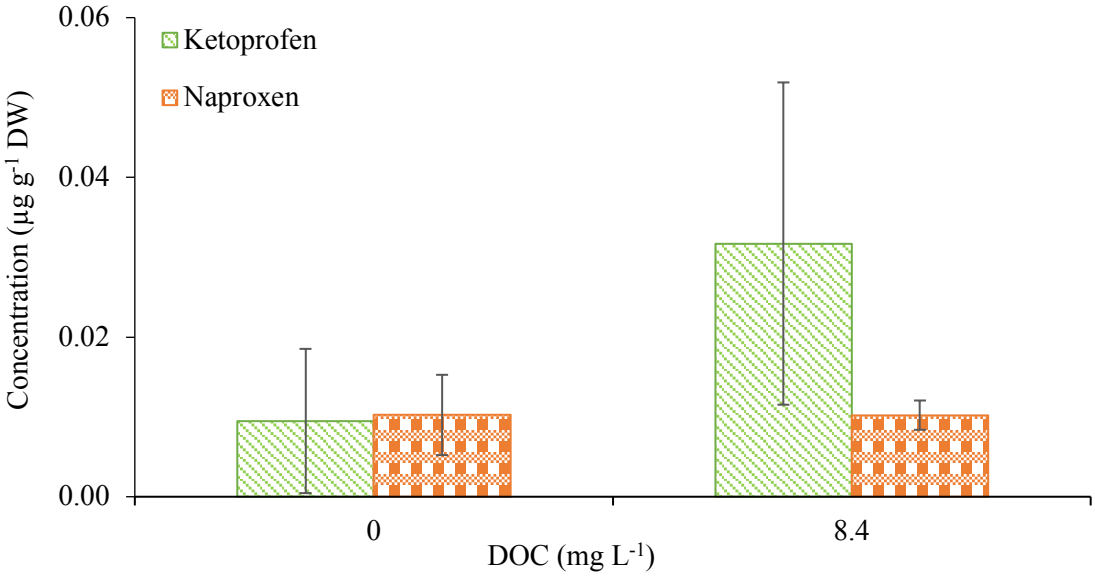

Figure 25. Ketoprofen and naproxen measured in the whole cucumber plant during the exposure period (48 h) in the absence (0 mg L^{-1}) and presence (8.4 mg DOC L^{-1}) of DOM. Mean values are shown (*n* = 3); bars represent standard errors.

Adsorption of Carbamazepine and Lamotrigine to Cucumber Roots

To evaluate the degree of CBZ and LTG adsorption onto cucumber roots, short-term (0-24 h) adsorption experiments were conducted on fresh and frozen-thawed roots. Carbamazepine, both as a single compound or in combination with LTG (bi-solute) resulted in a ratio between the final (C) and initial (C_0) concentration in the solution, C/C_0 ~1 throughout the sorption experiments for both the fresh and thawed roots. Since the root-to-water mass ratio was rather large (1:2 or 1:4) and the PPCPs concentrations were low (500 µg L^{-1} for each compound), the ratio of C/C_0 = 1 indicates that CBZ was not adsorbed to the external root tissue (**Fig. 26**). These results indicate that no partitioning into the root tissue occurred in the fresh roots, which may support the claim that CBZ uptake into plant roots is driven mainly by the transpiration stream.

In contrast to CBZ, LTG as a single-solute exhibited high sorption affinity to the fresh root, with 70% sorbed at 0.5 and 6 h, and a slight decline to 55% at 24 h. In bi-solute experiments, LTG

sorption was lower to the fresh roots, with 35% sorbed in 0.5 h, followed by an increase to 49% at 8 h and a slight decrease to 41% at 24 h (**Fig. 26a**). Lamotrigine sorption to thawed roots was similar in both treatments over time, with 58% and 46% at 0.5 h, 55% and 48% at 6 h and 44% and 43% at 24 h, in the single and bi-solute respectively (**Fig. 26b**).

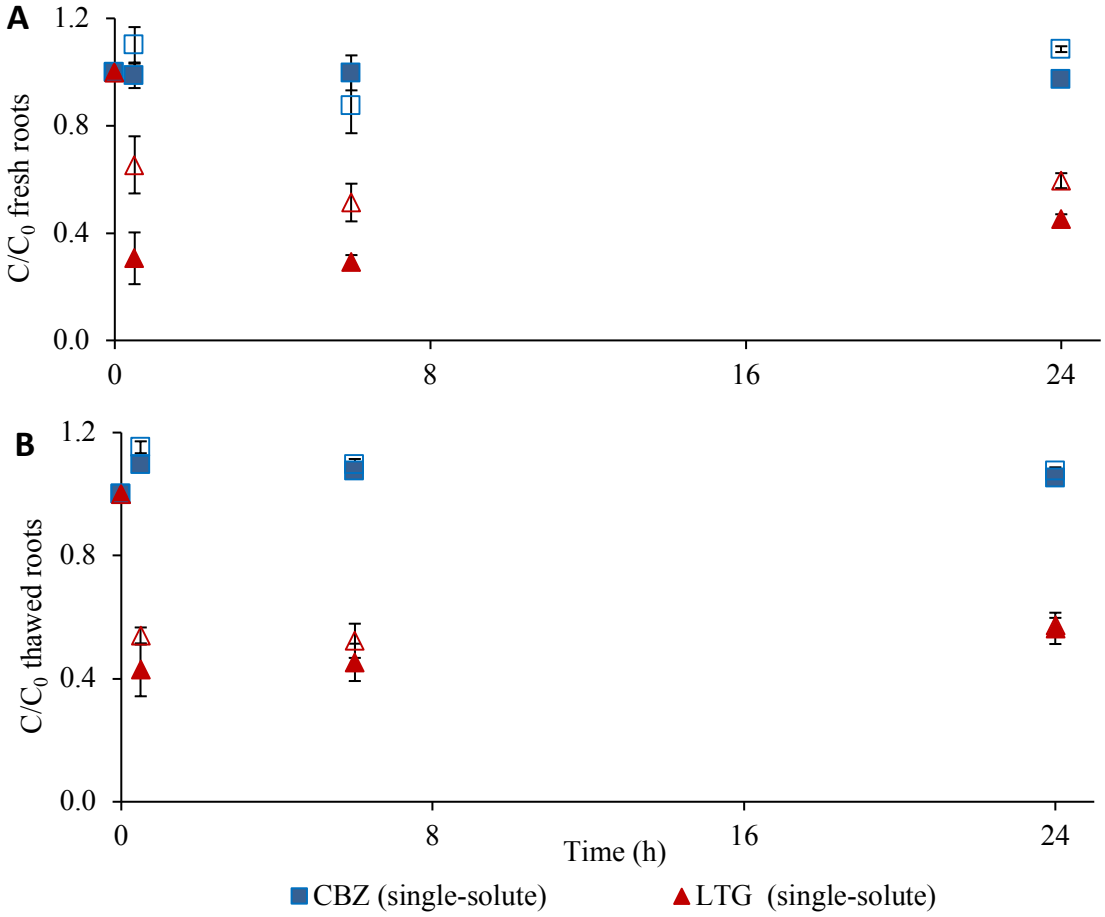

Figure 26. Sorption of carbamazepine (CBZ) and lamotrigine (LTG) to (A) fresh and (B) frozen and thawed cucumber roots. Final to initial solution concentrations (C/C_0) as both single (each compound individually) and bi-solute (mixture of both compounds). $C/C_0 = 1$ indicates no sorption, decreased C/C_0 indicate increased sorption. Mean values and standard errors ($n = 5$) are shown.

Kinetics of PPCP Uptake by Cucumber Plants

Carbamazepine Uptake Kinetics. We examined the kinetics of PPCP uptake from nutrient solution by cucumber. Carbamazepine uptake was not affected by the presence of LTG; therefore, the following discussion about root uptake combines data from the two treatments: single-solute (plants exposed only to CBZ) and bi-solute (plants exposed to both CBZ and LTG). For the CBZ single-solute system, CBZ concentration in the nutrient solution was ~1.72 μM for the first 48 h, after which a minor, but significant, rise in concentration was observed (1.98 μM at 72 h and 2.29 μM at 96 h). Similar trend (increase from 1.75 to 2.18 μm) was found in the bi-solute system.

Kinetic analysis of CBZ and water influx into the cucumber roots indicated that the initial uptake rate was high in the first 8 h, and decreased thereafter (**Fig. 27**). The water uptake rate strongly affected the CBZ-uptake rate, but this does not mean that CBZ was actually taken up with the water-influx stream. Alternatively, we suggest different routes for water and CBZ: while water uptake occurs mainly through regulated water channels (aquaporins) and is

affected by the plant's demand for water, the non-ionic CBZ molecule is mainly translocated via diffusion across root-cell membranes, and is thus largely affected by the concentration gradient across the membrane according to Fick's law.

To demonstrate the CBZ uptake mechanism, we calculated the apparent CBZ influx concentration (μM) as the ratio between CBZ uptake rate (μmol kg root^{-1} h^{-1}) and water uptake rate (L kg root^{-1} h^{-1}). A comparison of the apparent CBZ influx concentration to CBZ concentrations in the nutrient solution at each sampling time (**Fig. 28**) indicated that the initial (8 h) apparent CBZ influx concentration was higher than the CBZ concentration in the nutrient solution; thereafter, it was consistently lower than the CBZ concentration in the nutrient solution. This explains the increase in CBZ concentration in the nutrient solution with time. This was not due to rapid sorption of CBZ to the external root surfaces as demonstrated by the results presented in the previous section (**Fig. 26**). Moreover, transformation products of CBZ were not detected in the nutrient solution and adsorption to the glass jar or possible biotic and abiotic degradation was shown to not contribute to loss of CBZ from solution (data not shown). We suggest that the calculated apparent CBZ influx concentration truly represents CBZ influx across the root membrane (i.e., uptake). The higher initial influx of CBZ molecules through the root membrane, followed by a slower and steady influx rate probably indicate separate uptake mechanisms and routes for water and CBZ.

In contrast to the steady increase in CBZ concentration in the nutrient solution, CBZ concentration in the plant sap remained steady throughout the experiment (**Figs. 28 and 29**), and was slightly lower than the concentration measured in the nutrient solution. It is important to note that although further metabolism of CBZ occurred in the root, the concentrations of the detected metabolites (EP-CBZ, DiOH-CBZ, 2-OH-CBZ, and 3-OH-CBZ) in the roots were 1–3 orders of magnitude lower than that of CBZ (data not shown) and none of the metabolites was detected in the plant sap.

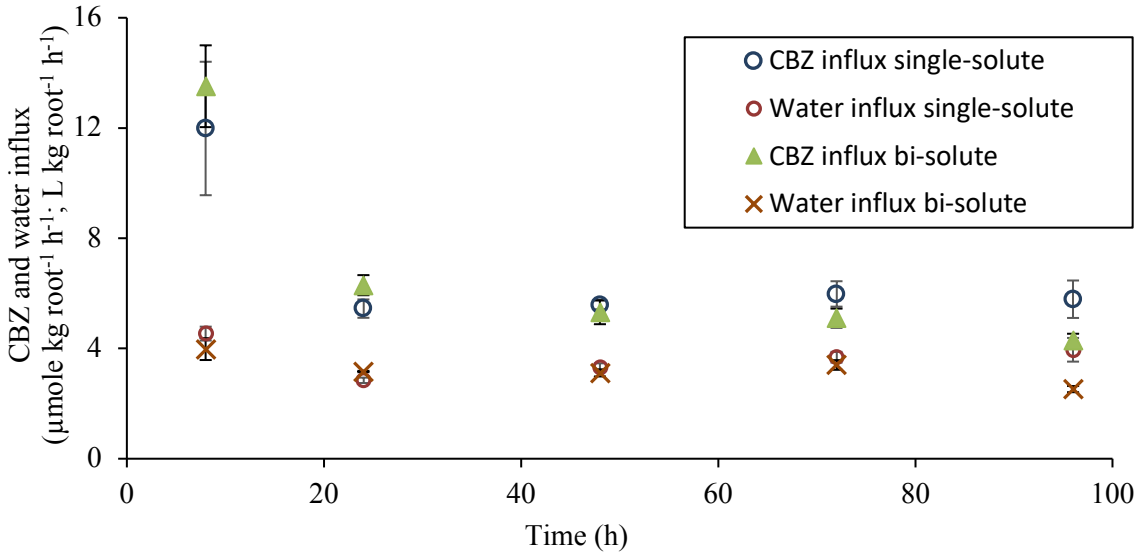

Figure 27. Carbamazepine (CBZ) and water uptake rates from the nutrient solution throughout the exposure period in the absence (single-solute) and presence (bi-solute) of lamotrigine. Data presented for dry weight. Mean values are presented; bars represent standard errors (*n* = 5).

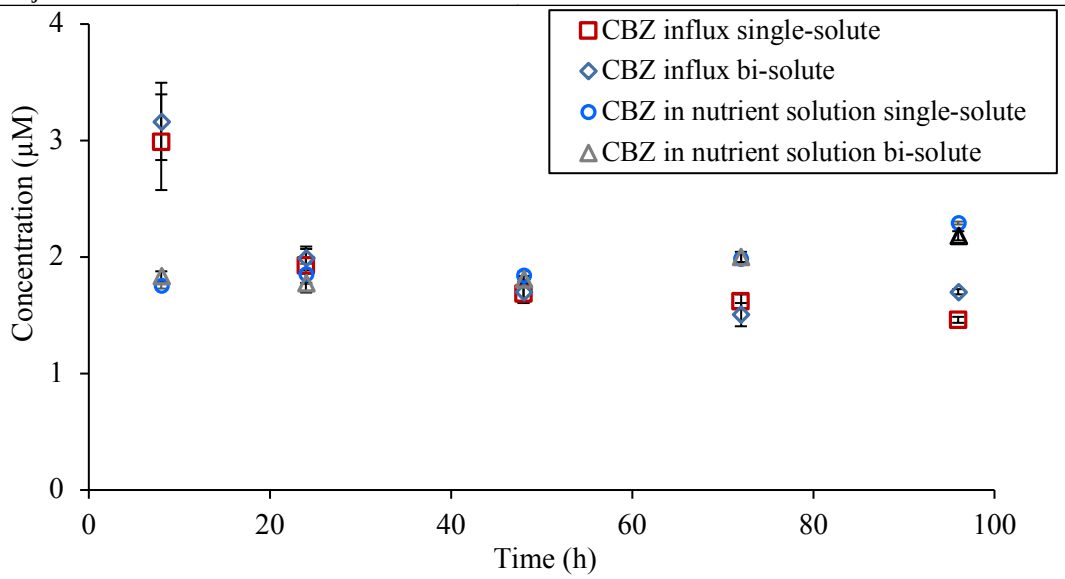

Figure 28. The apparent carbamazepine (CBZ) influx concentrations and the concentrations of CBZ in the nutrient solution in the absence (single-solute) and presence (bi-solute) of lamotrigine throughout the exposure period. Mean values are shown (*n* = 5); bars represent standard errors.

The transpiration stream concentration factor (TSCF, the ratio between the concentration in the sap and the concentration in the nutrient solution), for CBZ ($TSCF_{CBZ}$) in both the single- and the bi-solute systems was ~0.7 at 8 h, increased to 0.9 at 48 h, and declined to 0.7 at 96 h (**Fig. 29**).

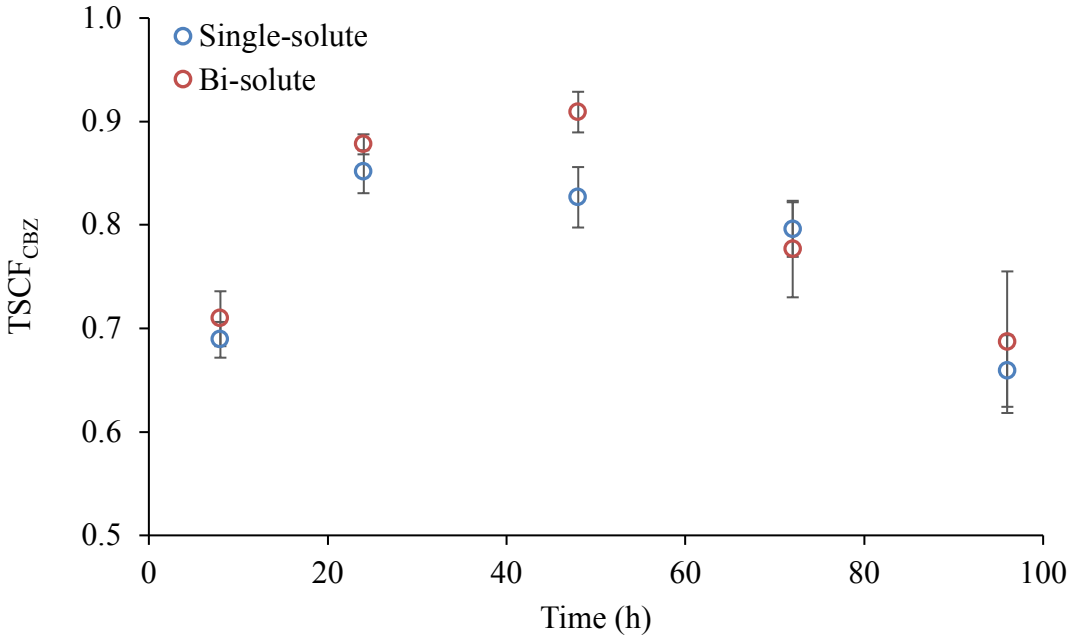

Figure 29. $TSCF_{CBZ}$ (transpiration stream concentration factor, calculated as the ratio between the carbamazepine concentration (CBZ) in the sap and the CBZ concentration in the nutrient solution) during the exposure period. Average data are presented for each treatments (in the presence, bi-solute, and absence, single-solute of lamotrigine) (*n* = 5). Bars represent standard errors.

Carbamazepine in the Plant Tissues. Concentrations of CBZ in root tissues (**Fig. 30**) were similar at 8 h for the single-solute and bi-solute systems, while the shoot concentration was

ten times lower. After 24 h, a sharp increase in CBZ concentration was visible in the shoots of both treatments, with a somewhat smaller increase in the roots. Slight, albeit significant difference between the two treatments (single- vs. bi-solute) was found at the 96 h sampling with a higher concentration of CBZ in the shoots in the single-solute system. This implies a degree of hindered translocation of CBZ in the bi-solute system. Accumulation of the sum of CBZ and its metabolites in the root (**Fig. 31**) exhibited the same trend as for the parent compound (**Fig. 30)**.

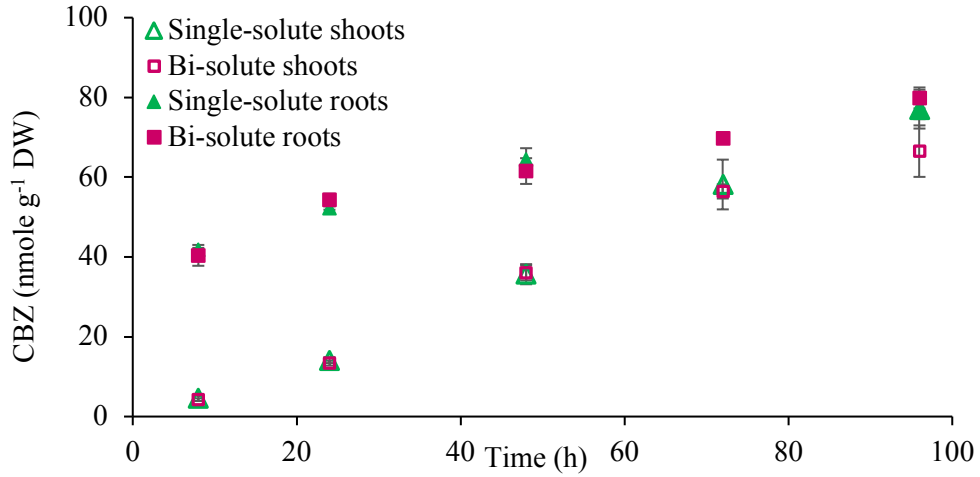

Figure 30. Carbamazepine (CBZ) concentrations in the root and shoot during the exposure period in the absence (single-solute) and presence (bi-solute) of LTG. Average data and standard errors are shown (*n* = 3).

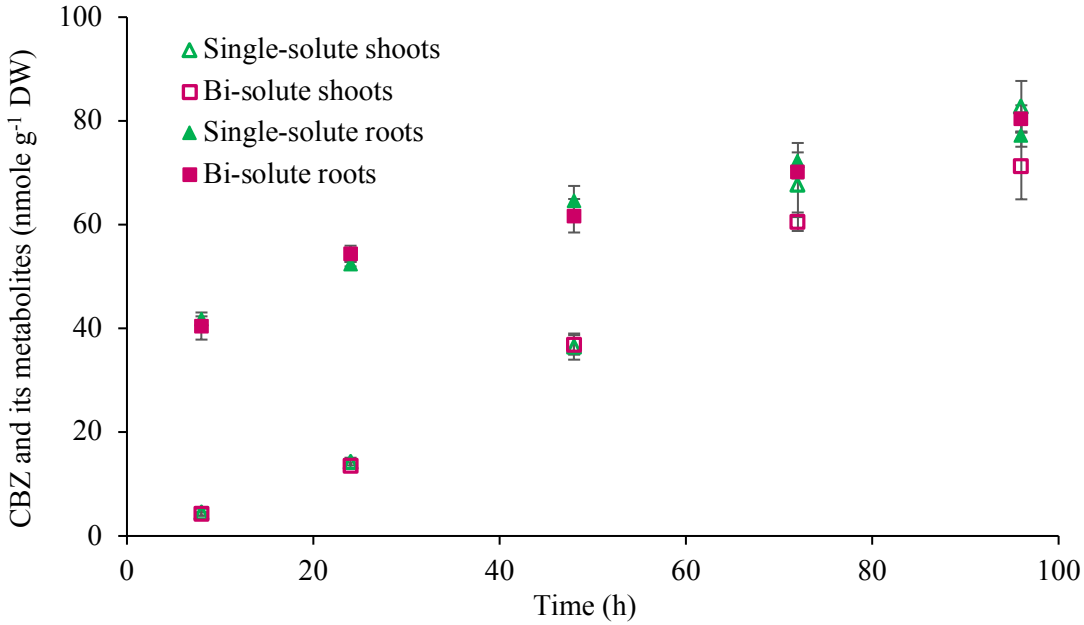

Figure 31. The sum of carbamazepine (CBZ) and its metabolites measured in the root and shoot during the exposure period in the absence (single-solute) and presence (bi-solute) of LTG. Average data and standard errors are shown (*n* = 3).

The trends of increased concentrations of CBZ and its metabolites in the shoot resulted in an increase in the shoot-to-root concentration ratio over time (**Fig. 32**). This increase was more pronounced in the single-solute system at 96 h because of higher concentrations of CBZ metabolites found in this treatment as compared to the bi-solute system (**Fig. 33**). Among the CBZ metabolites, we identified and quantified EP-CBZ, DiOH-CBZ, 2-OH-CBZ and 3-OH-CBZ in

the different plant compartments (**Fig. 33**). The concentrations of these metabolites were one to three orders of magnitude lower than that of CBZ. Of the detected metabolites, the 3-OH-CBZ concentration in the shoots and roots for both treatments was 2–3 times higher than observed for 2-OH-CBZ following the 48 h sampling. The concentration of DiOH-CBZ in the roots in the single-solute system at 72 h was higher than that in the bi-solute system. The DiOH-CBZ concentration was higher in the single-solute system in the shoots at both 72 and 96 h and in the roots at 96 h. We suggest that the relatively high concentrations of LTG in the roots in the bi-solute treatment resulted in lower DiOH-CBZ formation in the plants.

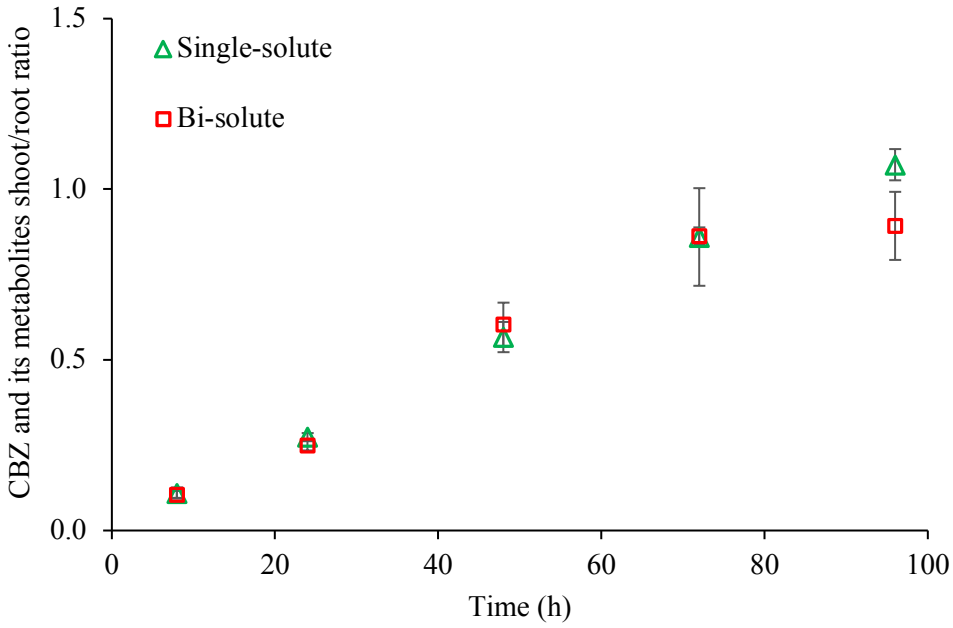

Figure 32. Concentration ratio of the sum of carbamazepine (CBZ) and its metabolites in the plant shoot to the sum in the roots during the exposure period in the absence (single solute) and presence (bi-solute) of LTG. Bars represent standard errors.

Our data may shed light on the location in the plant that CBZ is metabolized. We detected DiOH-CBZ and 3-OH-CBZ in the roots starting at 8 h, whereas in the shoot, these metabolites were not found until 48 h. We found EP-CBZ and 2-OH-CBZ in both plant parts at 8 h. DiOH-CBZ is produced from EP-CBZ via an epoxide hydrolase, and 3-OH-CBZ is derived directly from the parent compound. This suggests that the substrate for each metabolite may have been below the critical enzymatic activation concentration in the shoot during the first 24 h of exposure. Thereafter, shoot metabolism was far more pronounced than root metabolism, indicating that most of the metabolism of CBZ by CYP450 occurs in the shoot. In support of this, the ratio between EP-CBZ concentration in the shoots and CBZ concentration in the whole plant (roots + shoots) was higher and increased steadily with time (**Fig. 34**). Statistically identical results were obtained for both the single- and bi-solute systems.

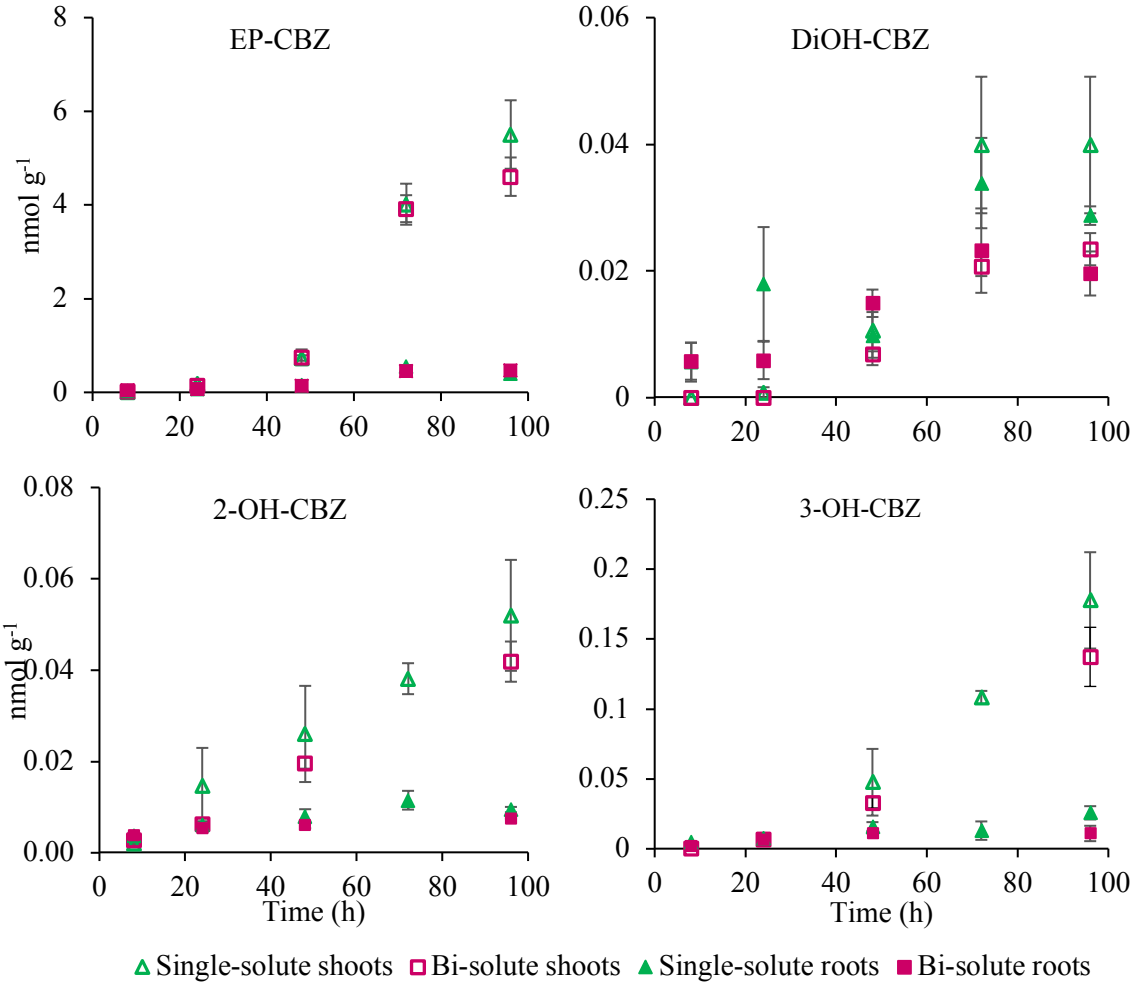

Figure 33. Carbamazepine (CBZ) metabolites EP-CBZ, DiOH-CBZ, 2-OH-CBZ and 3-OH-CBZ in the roots and shoots during the exposure period in the absence (single- solute) and presence (bi-solute) of LTG. Data presented for dry weight. Mean values are shown; bars represent standard errors.

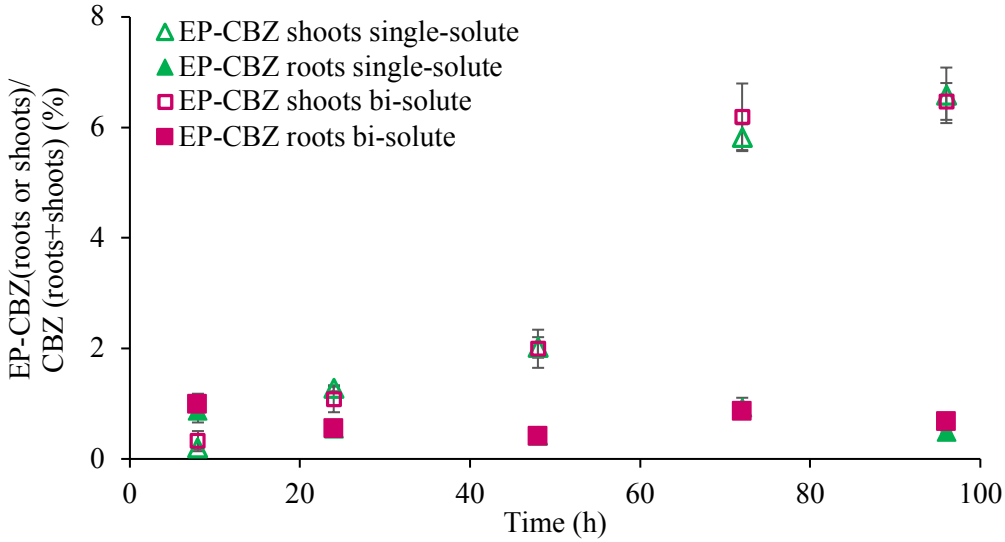

Figure 34. Ratio between EP-CBZ concentration in either roots or shoots and Carbamazepine (CBZ) concentration in the whole plant (roots + shoots) during the exposure period, in the absence (single- solute) and presence (bi-solute) of lamotrigine. Mean values and standard errors are shown.

Lamotrigine Uptake Kinetics. We examined the uptake kinetics of LTG in a similar manner as we did for CBZ. In single-solute experiments, LTG concentration in the nutrient solution increased slightly between 6 and 24 h, followed by a decline from 24 to 72 h (**Fig. 35**). In the bi-solute, the LTG external concentration decreased over time. The decrease in LTG concentration in the nutrient solution may be attributed to two separate mechanisms. The first, sorption to the roots, as demonstrated above in the root adsorption experiments (**Fig. 26a**). The second is ion trapping of LTG within the root vacuole (pH o~5.5) under which ~50% of LTG molecules are positively charged (similar mechanism suggested by Trapp[6]). Thus, decreasing the cytosolic LTG concentration and increasing its gradient between the external and internal compartments, increases the driving force for diffusion across the root membrane. The kinetics of LTG and water influx into the roots, calculated from the decreased amount in the nutrient solution and water uptake, are presented in **Fig. 36**. A higher LTG influx was found for the bi-solute system. Testing root adsorption to fresh cucumber roots also resulted in differences between the single- and bi-solute systems, where LTG root adsorption was lower in bi-solute system (**Fig. 26a**). The apparent LTG-influx was higher than the LTG concentration in the nutrient solution and higher than the apparent CBZ-influx concentration throughout the entire exposure period, although it declined over time (**Fig. 37**).

Plant tissue was analyzed for LTG in the bi-solute system only. In contrast to CBZ (**Fig. 30**), LTG exhibited higher accumulation in the cucumber roots, with a growing trend throughout the experiment. Mass balance calculation for LTG reviled a growing deficit over time (**Fig. 38**). The loss of LTG from the nutrient solution was not due to adsorption to the glass jar or possible biotic and abiotic degradation. We infer that this gap may be due to *in planta* metabolism. To date we have been unable to quantify LTG metabolites, yet can identify the presence of several of them in the plant material and propose probable atomic compositions in accordance to their measured masses (**Table 6**).

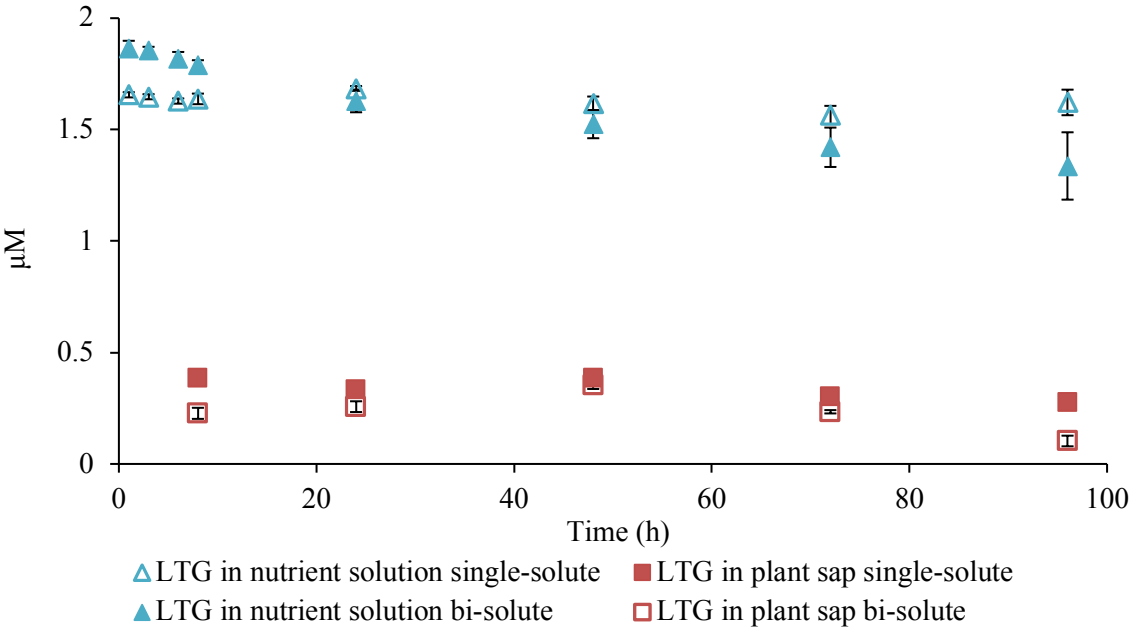

Figure 35. LTG concentration in the nutrient solution and in the plant sap during the exposure period in the absence (single-solute) and presence (bi-solute) of CBZ. Mean values and standard errors (*n* = 5) are shown.

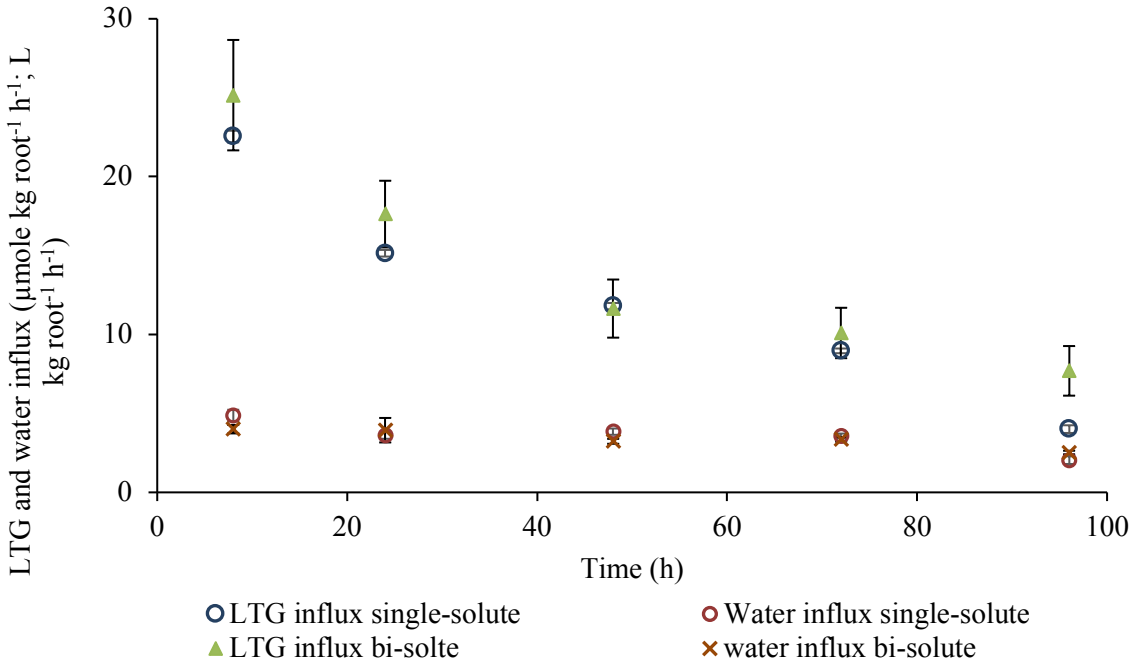

Figure 36. Lamotrigine (LTG) and water uptake rates from the nutrient solution throughout the exposure period. Mean values are presented for both the absence (single-solute) and presence (bi-solute) of CBZ; Data presented for fresh. Mean values and standard errors are presented (*n* = 5).

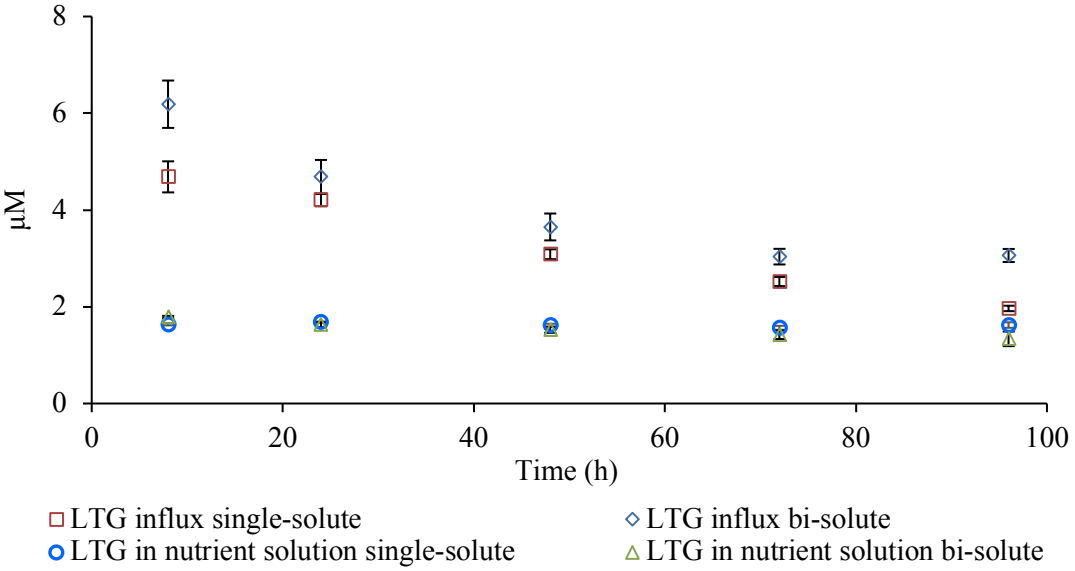

Figure 37. The apparent lamotrigine (LTG) influx concentrations and the concentrations of LTG in the nutrient solution in the absence (single-solute) and presence (bi-solute) of CBZ during the exposure period. Mean values and standard errors are shown (*n* = 5).

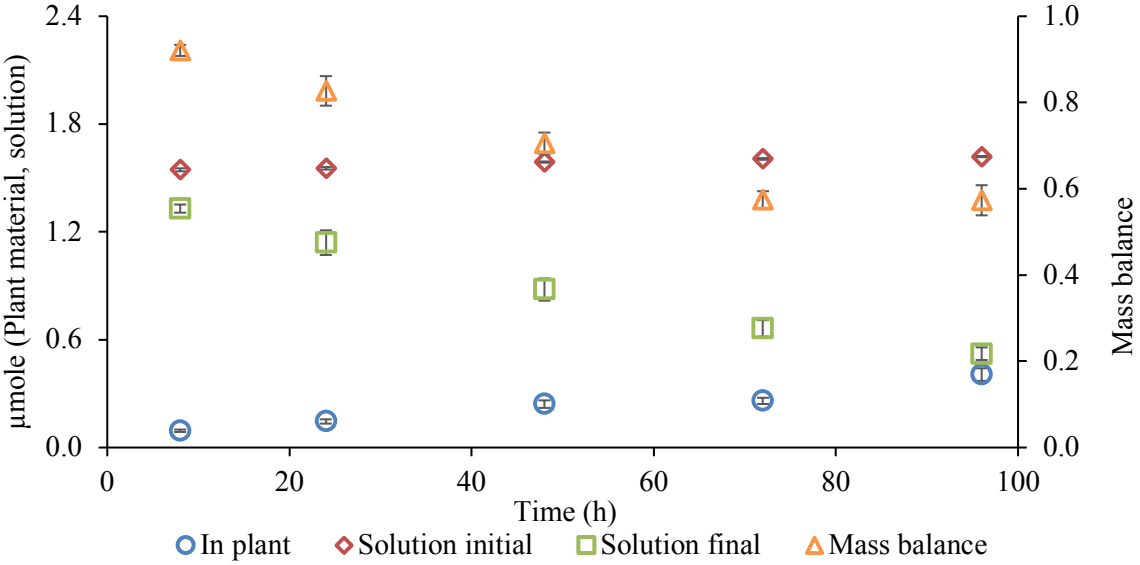

Figure 38. LTG amount (µmole) in the plant material (in plant), in the external solution at the start of the exposure period (solution initial; each sampling point consists of 5 replicas composed of different initial stock solutions), and in solution at each sampling time (solution final), and the calculated mass balance in the presence of CBZ (bi-solute). Average data are shown; bars represent standard errors (*n* = 3 for the plant material; *n* = 5 for the solution).

Table 6. Probable atomic composition of detected LTG metabolites in the plant material.

Retention Time (min)	Measured *m/z*	Atomic Composition
6.32	440.1080	$C_{19}H_{24}ON_5Cl_2S$
9.41	256.0150	$C_9H_8N_5Cl_2$
10.41	312.0412	$C_{12}H_{12}ON_5Cl_2$
11.97	362.0569	$C_{16}H_{14}ON_5Cl_2$
16.75, 17.08	394.1192	$C_{18}H_{22}ON_5Cl_2$

Uptake of Carbamazepine and Its Major Transformation Products

To assess whether the CBZ metabolites found in the plant tissues result from *in planta* metabolism or direct uptake of the metabolites from the irrigation water and/or soil solution, we tested the uptake of CBZ and its main metabolites for 48 h (**Fig. 39**). *In planta* metabolism differed depending on whether plants were exposed to CBZ or EP-CBZ. Uptake of EP-CBZ as the main substrate generated higher concentrations of the metabolites derived from the related metabolic pathways such as *cis*- and *trans*-DiOH-CBZ, acridine, 9,10-dihydro-9-oxoacridine-acridone and 9-acridinecarboxylic acid. Carbamazzepine as the main substrate yielded substantial amounts of EP-CBZ, 3-OH-CBZ at an order of magnitude lower concentration, and relatively low amounts of *trans*-DiOH-CBZ, 9,10-dihydro-9-oxoacridine-acridone and acridine-9-carboxylic acid. Both treatments exhibited a higher degree of metabolism in the roots, with higher concentration identified for all metabolites.

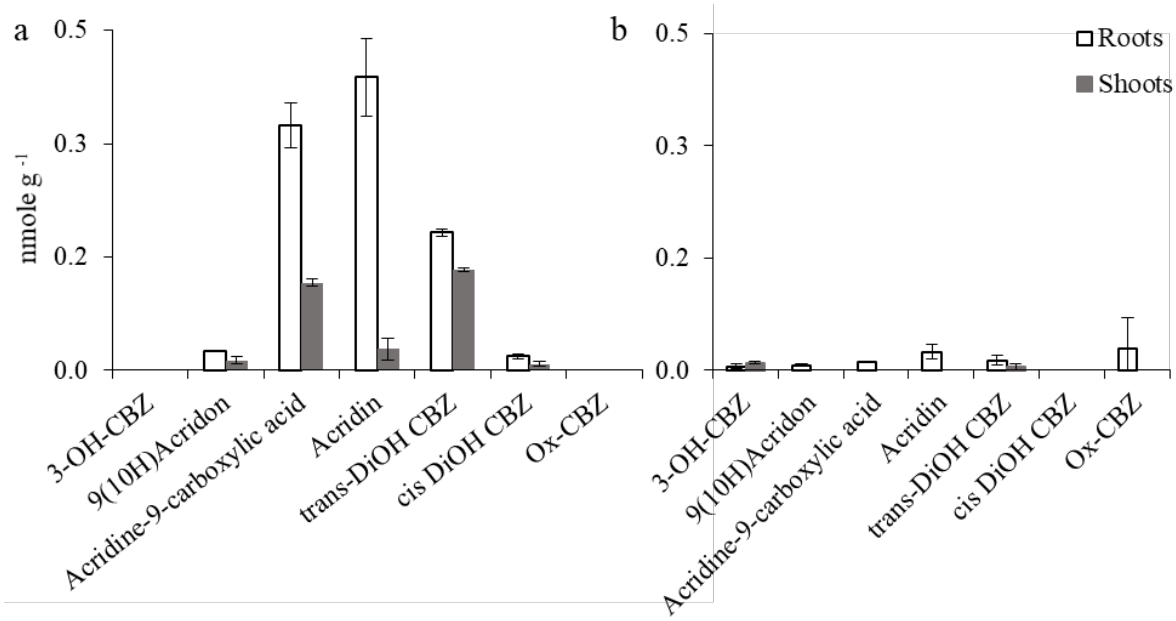

Figure 39. Metabolites of (a) EP-CBZ and (b) carbamazepine in cucumber roots and shoots after 48-h exposure. Both EP-CBZ and carbamazepine were introduced as a single solutes. Data presented for dry weight. Mean values and standard errors are shown (*n* = 3).

Phloem Sampling by the Sliverleaf Whitefly

To further understand *in planta* translocation mechanisms and to examine possible phloem translocation of PPCPs in the cucumber plant we incorporated phloem feeding silverleaf whitefly (*Bemisia tabaci*) into our hydroponic experiments. We analyzed the honeydew excreted by *Bemisia tabaci* that had fed on cucumber plants grown under hydroponic culture with either CBZ or LTG. We detected CBZ and LTG in the honeydew, demonstrating previously unreported translocation of CBZ and LTG into the plant phloem sap. We also found a small amount of EP-CBZ in some samples.

To distinguish between metabolites originating in the plant phloem and those produced by the whitefly, *Bemisia tabaci* adults were exposed to CBZ under artificial feeding conditions. In these experiments, we found EP-CBZ, 2-OH-CBZ, and 3-OH-CBZ in the honeydew, demonstrating the ability of the whitefly to metabolize CBZ. At present, we are unable to conclude whether EP-CBZ detected in the honeydew originates from metabolism within the plant or within the fly.

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Changes to Original Research Plan

As noted in a previous annual report, we aimed to systematically evaluate the bioaccumulation of chemicals of emerging concern with contrasting chemical characteristics by the model plant *Arabidopsis thaliana* as well as by crop species. Our initial objectives were to: (a) determine the influence of chemical properties on uptake and translocation pathways in selected plants; (b) relate plant root physiology to chemical uptake; and (c) elucidate the effects of soil properties on chemical availability, uptake, and distribution in selected plants. We have shown that chemical molecular descriptors alone have limited ability to adequately predict uptake/bioaccumulation (Miller et al. Environ. Sci. Technol. 2016)[1] and predictive efforts using existing data may be hindered by inadequate understanding of metabolism in planta and effects of contaminants on plants. We therefore adjusted the focus of our objectives accordingly to include studies on *in planta* metabolism of wastewater-derived micropollutants and the effects of exposure to mixtures of these compounds on uptake and accumulation.

Publications for Project US-4771-14R

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun
Published	Reviewed	*Miller, E. L.; Nason, S. L.; Karthikeyan, K. G.; Pedersen, J. A.*	Root uptake of pharmaceuticals and personal care products	***Environmental Science and Technology***	50 : 525-541 2016	US only
Submitted	Reviewed	*Nason, S. L.; Miller, E. L.; Karthikeyan, K. G.; Pedersen, J. A.*	Plant-induced changes to rhizosphere pH influence uptake of ionizable organic contaminants	***Environmental Science & Technology Letters***	: 2018	US only
Published	Thesis - Ph.D.	*Nason, S. L.*	Plant accumulation of pharmaceuticals		: 2017	US only
Published	Thesis - Ph.D.	*Goldstein, M.*	Uptake and fate of pharmaceuticals in vegetable plants		: 2017	IS only
Published	Abstract - Presentation	*Miller, E. L. Nason, S. R.; Karthikeyan, K. G.; Pedersen, J. A.*	Rhizosphere effects on lamotrigine accumulation by wheat plants	***Abstracts Papers Amer. Chem. Soc.***	: 2018	US only
Published	Abstract - Presentation	*Nason, S.; Miller, E. L.; Karthikeyan, K. G.; Pedersen, J. A.*	Model systems to study plant accumulation of ionizable organic contaminants	***Abstracts Papers Amer. Chem. Soc.***	: 2017	US only
Published	Abstract - Presentation	*Pedersen, J. A.*	Plant uptake of pharmaceuticals: Insights from model plants	***ASA/CSSA/SSSA***	: 2016	US only
Published	Abstract - Presentation	*Pedersen, J. A.; Miller, E. L.; Nason, S.; Karthikeyan, K. G.*	Phytotoxicity of pharmaceuticals and personal care product ingredients	***ASA/CSSA/SSSA***	: 2015	US only